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14. ABSTRACT

By facilitating the structural maturation and thereby the stability and activity oncogenic proteins, such as ErbB2, the molecular chaperone Hsp90 has emerged as a promising cancer therapeutic target. Toward understanding the regulation of Hsp90 and identifying new therapeutic approach targeting Hsp90 activity, we have characterized reversible acetylation as a critical mechanism that regulates Hsp90 function. Here we present evidence that Hsp90 chaperone activity is regulated by reversible acetylation and controlled by the deacetylase HDAC6. Inactivation of HDAC6 leads to Hsp90 hyperacetylation, its dissociation from an essential co-chaperone, p23, and a loss of chaperone activity. Using glucocorticoid receptor (GR) as a model client protein, we showed that in HDAC6 deficient cells, Hsp90-dependent maturation of the glucocorticoid receptor (GR) is compromised, providing evidence that HDAC6-catalyzed deacetylation is critical for Hsp90 activity. Our study identifies reversible acetylation as a unique mechanism that regulates Hsp90 chaperone complex activity. Based on this observation, we are now investigating whether HDAC6-regulated Hsp90 acetylation is also critical for ErbB2-induced tumor transformation.

15. SUBJECT TERMS
p53, acetylation, ubiquitination,MDMD2, HDAC

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Introduction:

The long-term goal of this research proposal is to test the hypothesis that HDAC6-regulated acetylation plays a critical role in Hsp90-dependent oncogenic signaling including those associated with ErbB2 and related EGFR. We wish to determine if inactivation of HDAC6 could serve as a therapeutic target for treating breast tumor.

While gene amplification is the causative event underlying over-expression of ErbB2, the stability and activity of this oncoprotein critically depends on the molecular chaperone heat shock protein 90 (Hsp90). In conjunction with specific cofactors, termed co-chaperones, Hsp90 is believed to facilitate the proper folding of mutated, chimeric or over-expressed oncogenic proteins such as ErbB2, thereby promoting malignant transformation. Supporting this view, Hsp90 is abnormally up-regulated in many human tumors including breast cancers, and specific Hsp90 inhibitors, such as 17-allylamino-17-demethoxygeldanamycin (17-AGG), induce destabilization of ErbB2 and show anti-tumor activity against human breast cancer in murine xenograft models. While Hsp90 has emerged as a promising target in cancer treatment, little is known about how its activity is regulated to facilitate ErbB2-mediated oncogenesis. Understanding this process could provide valuable information for developing a mean to disrupt such regulation and thereby impair ErbB2-dependent tumorigenesis. Here we have identified HDAC6-regualted reversible acetylation as a critical mechanism that regulates Hsp90 chaperone activity. We demonstrated that HDAC6 is a Hsp90 deacetylase. Furthermore, using a model Hsp90 client protein glucocorticoid receptor, we demonstrated that HDAC6 is required for full Hsp90 chaperone activity. This observation provides strong rationale to study the potential role for HDAC6 in ErbB2-induced tumor transformation as well as other oncogenic signaling events known to be affected by Hsp90. Indeed, our study supports the idea that HDAC6 is required for ErbB2-tumor transforming activity both in vitro and vivo. Lastly, we have discovered that HDAC6 is important for macropinocytosis, which has been intimately linked to oncogenic signaling and cell migration related to metastasis.

Body:

1. **Hsp90 chaperone activity toward glucocorticoid receptor is defective in cells deficient in HDAC6 (see attached manuscript)**
2. **HDAC6 is required for ErbB2-dependent signaling and efficient proliferation in a xenograft model.**
3. **HDAC6 regulates macropinocytosis via regulating Hsp90.**

1. We have showed that Hsp90 becomes hyperacetylated in HDAC6 deficient cells. The hyperacetylation of Hsp90 is correlated with a defect in the maturation of glucocorticoid receptor (GR). In the current study, in collaboration with Dr. William Pratt's group, we now provided the critical evidence that Hsp90 produced in HDAC6 deficient cells is in fact defective in supporting in vitro GR maturation, and most importantly, this deficiency can be reversed by wild type Hsp90 purified from wild type cells. (please see attached PDFs for detaials)

2. HDAC6 and ErbB2-dependent oncogenic transformation

A. As ErbB2-maturation also depends on Hsp90, we test the hypothesis that HDAC6-mediated deacetylation is important for Hsp90 to efficiently facilitate ErbB2-depenent oncogenesis. We show that inactivation of HDAC6 by small interference RNA markedly reverses the transformed phenotype of SKBR3 and SKOV3, two ErbB2-overexpressing human breast cancer cell line (Figure 1). This is assessed by anchorage independent growth (Figure 1A, B) and xenograft tumor model (Figure 1C, D) Importantly, this phenotype can be reversed by stably reintroduction of a siRNA resistant wild type HDAC6 (WT, Panel B, C, D) but not catalytically inactive HDAC6 mutant (CD). These studies clearly establish HDAC6 as essential for robust anchorage independent proliferation and tumor proliferation.

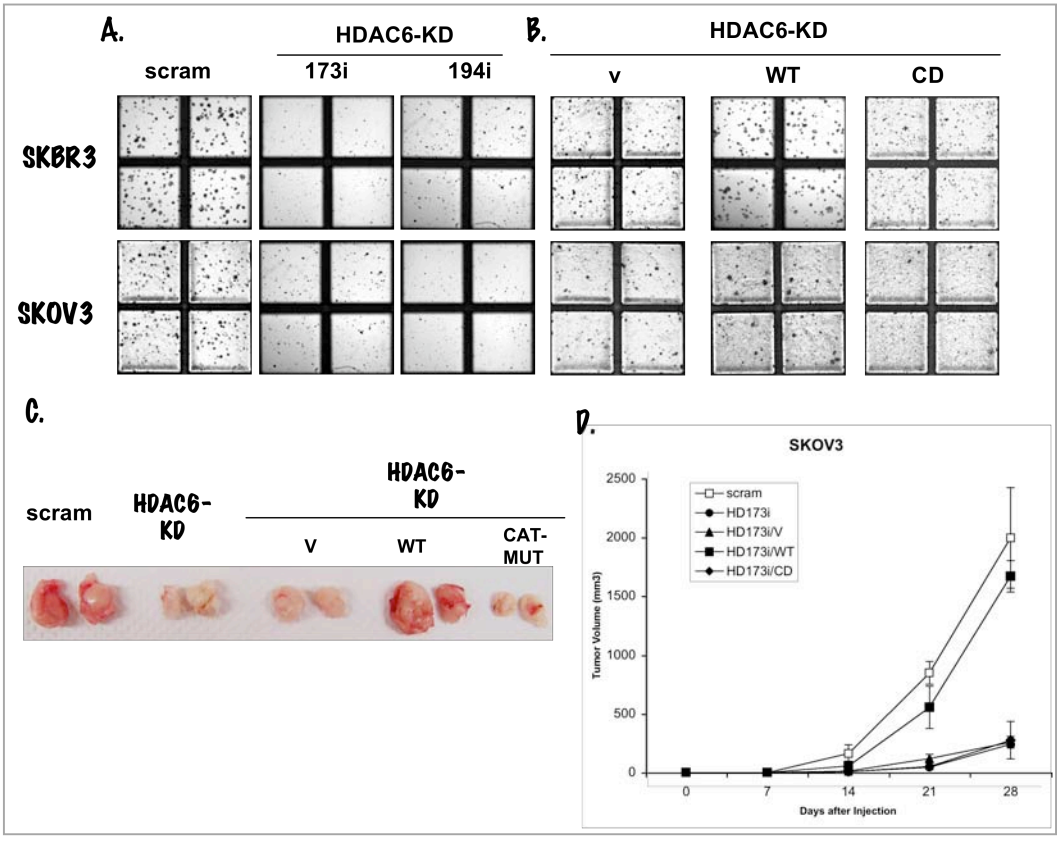

Figure 1. Anchorage independent growth is impaired with loss of HDAC6 in SKBR3 and SOV3 erb2-over-expressing cells. (A) SKBR3 or SKOV3 cells stably expressing two independent HDAC6 specific siRNA show a significantly lower number of colony formation on soft agar. (B) HDAC6 knockdown (KD) stably expressing siRNA resistant wild type and catalytic inactive (CD) HDAC6 mutant. Note that reintroduction of

wild type HDAC6 can restore colony formation. (C-D) 10^5 of SKOV3 or HDAC6 knockdown, or HDAC6 KD reconstituted with wild type or catalytically inactive HDAC6 were injected into immunodeficient nude mice. The tumor growth was evaluated in C (gross morphology) and D (size). Note that HDAC6 KD cells formed much smaller tumors and this phenotype can be reversed by the reintroduction of wild type but not catalytically inactive mutant.

B. To investigate the basis for the requirement for HDAC6 in efficient tumor formation, we assessed the proliferation index of HDAC6 knockdown tumors. As shown in Figure 2, HDAC6 KD tumors show fewer Ki63 positive cells indicating that HDAC6 is required for efficient tumor cell proliferation. Consistent with a proliferation defect, the biochemical evidence that erbB2-dependent erk Kinase phosphorylation is defective in HDAC6 deficient cells (Figure 3).

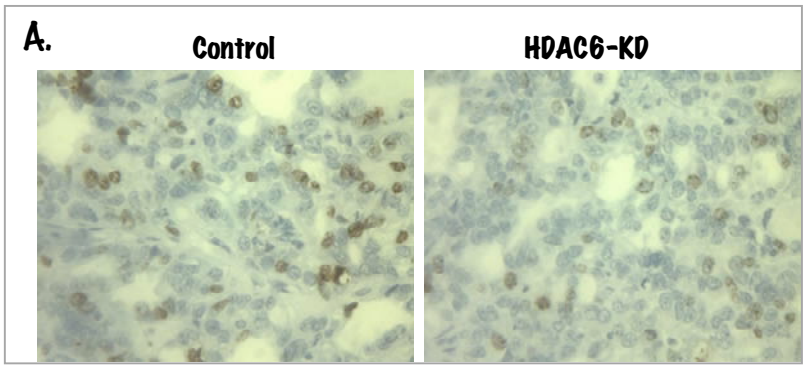

Figure 2. Tumors from a control or HDAC6 knockdown SKOV3 were sectioned and stained for Ki67. Note that there is a significant decrease in the number of Ki67 positive cells in HDAC6 knockdown tumors.

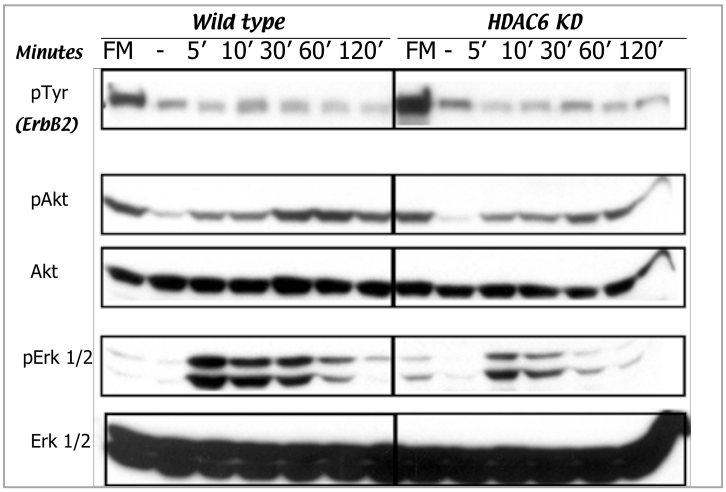

Figure 3. Wild type or HDAC6 knockdown skbr3 cells are cultured in full medium (FM), of serum starved followed by EGF stimulation. Note that phospho-ErbB2 (pTyr), phospo-AKT (pAKT) appear to be comparable in wild type and HDAC6 KD cells. However, phospho-Erk1/2 (p-Erk1/2) is dramatically reduced in HDAC KD cells.

3. HDAC6 regulates macropinocytosis via Hsp90

Macropinocytosis is normally regulated by growth factor, such as PDGF and EGF. Importantly, macropinocytosis often becomes constitutive upon oncogenic transformation by oncogenic ras, src and PI3K. Macropinocytosis might contribute the robust growth of tumors and motility associated with metastasis. We showed that HDAC6 and Hsp90 are both essential for efficient macropinocytosis. This new findings provide additional pathway by which HDAC6 might promote oncogenic transformation (see attached PDF).

Key Research Accomplishment

1. We have identified HDAC6 as a Hsp90 deacetylase and show that Hsp90 acetylation negatively regulates its chaperone activity by dissociating its essential co-factor.
2. We have established the preliminary result that HDAC6 is required for Hsp90-dependent ErbB2 oncogenic activity and identified another potential defect in raf-1 and B-Raf kinase stability.
3. We have demonstrated that HDAC6 is critical for cell migration and macropinocytosis induced by growth factor. This effect is mediated at least in part by Hsp90. This part of study is now submitted for publication (see attached PDF file).

Reportable Outcome:

Kovacs, J.J., Murphy, P. J., Stéphanie Gaillard, Zhao, X., Wu, J.-T., Nicchitta, C., Yoshida, M., Toft, D., Pratt, W., and **Yao, T.-P**. The deacetylase HDAC6 regulates Hsp90 acetylation and chaperone - dependent activation of glucocorticoid receptor. **Molecular Cell** 18(5) 601-607 (2005)

Murphy P.J, Morishima Y, Kovacs J.J, **Yao T.-P**, and Pratt WB. Regulation of the dynamics of hsp90 action on the glucocorticoid receptor by acetylation/deacetylation of the chaperone. **J. Biol. Chem.** 280(40):33792-33799 (2005).

Kovacs, J.J., Cohen T and **Yao, T.-P.** Chaperoning steroid hormone signaling via reversible acetylation. Nucl Recept Signal. 3:e004 (2005) (review article)

Gao, Y.-S., Hubbert, C., Lee, Y.-S., Kovacs, J. J., Lu, J., and **Yao, T.-P.** The microtubule-associated deacetylase HDAC6 regulates growth factor induced actin cytoskeleton remodeling and macropinocytosis (**under revision**)

Conclusion:

Our study has provided strong evidence that Hsp90 chaperone activity is regulated by reversible acetylation controlled by HDAC6. This result provides strong rationale to investigate the role for HDAC6 in ErbB2-dependent and/or Hsp90 dependent oncogenic transformation.

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HDAC6 Regulates Hsp90 Acetylation and Chaperone-Dependent Activation of Glucocorticoid Receptor

Short Article

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Summary

The molecular chaperone heat shock protein 90 (Hsp90) and its accessory cochaperones function by facilitating the structural maturation and complex assembly of client proteins, including steroid hormone receptors and selected kinases. By promoting the activity and stability of these signaling proteins, Hsp90 has emerged as a critical modulator in cell signaling. Here, we present evidence that Hsp90 chaperone activity is regulated by reversible acetylation and controlled by the deacetylase HDAC6. We show that HDAC6 functions as an Hsp90 deacetylase. Inactivation of HDAC6 leads to Hsp90 hyperacetylation, its dissociation from an essential cochaperone, p23, and a loss of chaperone activity. In HDAC6-deficient cells, Hsp90-dependent maturation of the glucocorticoid receptor (GR) is compromised, resulting in GR defective in ligand binding, nuclear translocation, and transcriptional activation. Our results identify Hsp90 as a target of HDAC6 and suggest reversible acetylation as a unique mechanism that regulates Hsp90 chaperone complex activity.

Introduction

The heat shock protein Hsp90 and its cofactors form molecular chaperone complexes that facilitate the structural maturation of its substrates, termed client proteins. The Hsp90-assisted maturation of client proteins often leads to an enhanced activity and stability. Although initially recognized as a stress-induced protein, the realization that many Hsp90 targets are critical for normal and oncogenic signaling has identified Hsp90 as an important modulator in cell signaling and a promising target in cancer therapy (reviewed in Neckers [2002] and Pratt and Toft [2003]). The emerging significance of Hsp90 in both normal and oncogenic signaling underlines the fundamental importance of understanding how Hsp90 activity is regulated.

Prominent examples of Hsp90 client proteins include steroid hormone receptors and kinases important for oncogenesis (Richter and Buchner, 2001). Among them, the Hsp90-dependent maturation of GR, a member of the steroid hormone receptor family, is best characterized. GR mediates biological effects of glucocorticoid by acting as a transcription factor (Giguere et al., 1986). Upon binding to glucocorticoid, GR becomes activated and translocates into the nucleus where it controls specific transcriptional programs. In the absence of its ligand, however, GR is inactive and resides in the cytoplasm where it associates with Hsp90 (Cadepond et al., 1991). It has been shown that the association with Hsp90 is critical for GR to assume a competent ligand binding conformation. In vitro and in vivo analyses demonstrate that Hsp90, in conjunction with a selected set of cochaperone proteins, is required for GR to bind hormone with high affinity (reviewed in Pratt and Toft [2003]). The study of Hsp90-dependent GR maturation has provided mechanistic insight into the basic steps of chaperone complex-client protein assembly and the important functions of cochaperones (Dittmar et al., 1997). However, the critical question regarding whether and how Hsp90 is regulated in these processes is poorly understood.

Reversible protein acetylation has been characteristically linked to histone and chromatin-dependent processes. Recent studies, however, have revealed a much broader array of biological processes that involve protein acetylation (reviewed in Cohen and Yao [2004]). For example, the characterization of the deacetylase HDAC6, a member of the histone deacetylase family, has implicated protein acetylation in the regulation of microtubules, growth factor-induced chemotaxis, and the processing of misfolded protein aggregates (Haggarty et al., 2003; Hubbert et al., 2002; Kawaguchi et al., 2003; Matsuyama et al., 2002; Zhang et al., 2003). Consistent with these apparently nongenomic functions, HDAC6 is mainly localized to the cytoplasm (Hubbert et al., 2002; Verdel et al., 2000). These observations suggest reversible protein acetylation controlled by HDAC6 might have important regulatory roles in cytoplasmic-based processes. Identifying new substrates for HDAC6 will be a critical step toward deciphering the general significance of protein acetylation in the cytoplasm.

In this report, we have demonstrated that Hsp90 is a substrate of HDAC6, and its chaperone activity is regulated by acetylation. We have shown that HDAC6 and Hsp90 form a complex in vivo and that HDAC6 functions as an Hsp90 deacetylase. Inactivation of HDAC6 by pharmacological inhibitor or by specific siRNA leads to hyperacetylation of Hsp90, the dissociation of an

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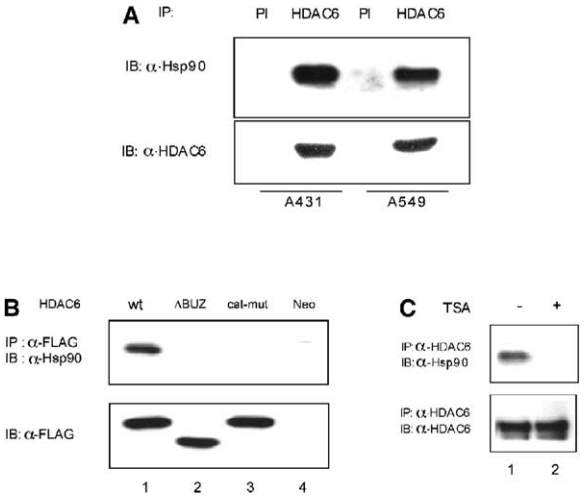

Figure 1. HDAC6 Associates with Hsp90

(A) A431 or A549 cell lysates were immunoprecipitated with α-HDAC6 antibody or preimmune serum (PI) and immunoblotted for Hsp90.

(B) Cell lysates from NIH-3T3 cell lines stably overexpressing FLAG-HDAC6, FLAG-HDAC6-ΔBUZ (ubiquitin binding deficient mutant), FLAG-HDAC6-cat-mut (H216/611A, catalytically inactive mutant), or Neo vector control were immunoprecipitated with α-FLAG antibody and blotted with α-Hsp90.

(C) A549 cells were left untreated or subjected to a 4 hr treatment of trichostatin A (TSA) (1 μM). Cell lysates were then immunoprecipitated with α-HDAC6 antibody and then blotted for Hsp90.

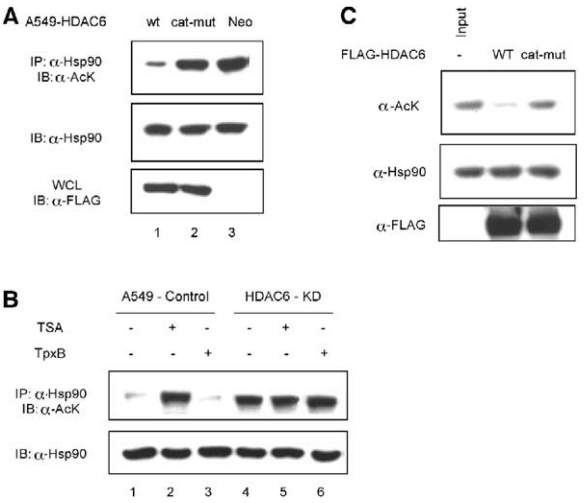

Figure 2. HDAC6 Regulates the Deacetylation of Hsp90

(A) Lysates from A549 cell lines stably overexpressing wild-type (wt), catalytically inactive mutant HDAC6 (cat-mut), or Neo vector control were immunoprecipitated with α-Hsp90 and then immunoblotted with α-acetylated lysine antibody (α-AcK).

(B) A549 cells stably expressing pSuper control plasmid (wt) or HDAC6 siRNA (KD) were left untreated, treated for 4 hr with TSA, or treated for 4 hr with TPXb (100 nM). Cell lysates were then immunoprecipitated with α-Hsp90 followed by immunoblotting with α-AcK antibody. Both HDAC6 siRNA and TSA treatment cause a dramatic rise in the level of acetylated Hsp90.

(C) A549 cells were treated with TSA for 8 hr to induce an acetylated population of Hsp90. Hsp90 was immunoprecipitated from cell lysates and immunoblotted with α-AcK to show an enrichment of acetylated Hsp90 (Input). 293T cells were transfected with FLAG-tagged wt or catalytically dead HDAC6. These cells were lysed, and wt or cat-dead HDAC6 was immunprecipitated with α-FLAG antibody. The purified Hsp90 was then incubated with the purified FLAG-HDAC6 wt or cat-dead protein at 37°C for 60 min. The reactions were then subjected to SDS-PAGE and immunoblotted with the indicated antibodies.

essential cochaperone, p23, from Hsp90, and a loss of chaperone activity toward GR. We found that GR in HDAC6-deficient cells is defective in ligand binding, nuclear translocation, and gene activation, revealing a failure of chaperone-dependent maturation in the absence of functional HDAC6. Our results identify the Hsp90-GR complex as a target of HDAC6 and suggest reversible acetylation as an important mechanism in regulating Hsp90 molecular chaperone activity.

Results

HDAC6 Associates with Hsp90 In Vivo

To gain further insight into protein acetylation in the cytoplasm, we searched for cellular targets for HDAC6. By using an affinity trap approach, we identified Hsp90 as a prominent HDAC6 interacting partner by both mass spectrometry (data not shown) and direct immunoprecipitation, which shows that endogenous HDAC6 and Hsp90 can be abundantly and specifically coimmunoprecipitated from multiple cell lines (Figure 1A). To further characterize the HDAC6-Hsp90 interaction, we assessed whether mutations or pharmacological inhibitors that affect HDAC6 activity would influence its association with Hsp90. Full HDAC6 function requires both its deacetylase activity and ubiquitin binding activity, which is mediated by a unique zinc finger, termed the BUZ finger (Hubbert et al., 2002; Kawaguchi et al., 2003). We found that inactivation of HDAC6 either by mutations or by treatment with the inhibitor trichostatin A (TSA) led to the dissociation of HDAC6 from Hsp90 (Figure 1B, compare lanes 1 and 3 and Figure 1C). Furthermore, an HDAC6 mutant lacking the ubiquitin binding BUZ finger also fails to bind Hsp90 efficiently (Figure 1B, lane 2). These data show that the HDAC6-Hsp90 interaction is specific and requires both deacetylase and ubiquitin binding activities of HDAC6.

HDAC6 Regulates Hsp90 Acetylation

An interaction between HDAC6 and Hsp90 is significant, as Hsp90 was recently reported to be an acetylated protein (Yu et al., 2002). We thus investigated the possibility that HDAC6 functions as an Hsp90 deacetylase. To this end, we first determined whether overexpression of HDAC6 can lead to Hsp90 deacetylation in vivo. As shown in Figure 2A (lane 3), a basal level of Hsp90 acetylation can be detected in control A549 cell lines. Hsp90 acetylation levels, however, are markedly reduced in A549 cells that stably overexpress wild-type (wt), but not a catalytically inactive mutant, HDAC6 (Figure 2A, compare lanes 1 and 2). Conversely, in A549 cells stably expressing siRNA that reduces HDAC6 expression (HDAC6 knockdown [Kawaguchi et al., 2003]), Hsp90 acetylation levels are substantially increased (Figure 2B, compare lanes 1 and 4). These results show that HDAC6 can function as an Hsp90 deacetylase in

vivo. Additionally, we found that TSA, but not TPXb, a potent inhibitor for all HDAC members except HDAC6 (Furumai et al., 2001), also induces potent Hsp90 acetylation (compare Figure 2B, lanes 1–3). We note that TSA treatment has little effect on Hsp90 acetylation in HDAC6 knockdown cells, indicating that HDAC6 is the primary TSA-sensitive endogenous Hsp90 deacetylase (compare Figure 2B, lanes 4–6). Importantly, immunopurified wt, but not catalytically inactive mutant, HDAC6 can efficiently deacetylate acetylated Hsp90 in vitro as well (Figure 2C). Together, these results demonstrate that HDAC6 has Hsp90 deacetylase activity.

Chaperone-Dependent GR Maturation Is Defective in HDAC6-Deficient Cells

We next determined if HDAC6-regulated Hsp90 acetylation is important for Hsp90 chaperone function. The requirement of Hsp90 chaperone activity for efficient ligand binding and the subsequent activation and nuclear translocation of the GR are the most well-characterized functions for Hsp90. To establish whether acetylation is important for Hsp90-dependent GR ligand binding, cytosols prepared from control and HDAC6 knockdown 293T cells were incubated with radiolabeled dexamethasone, and steroid binding to the GR was determined. As expected, endogenous GR from control 293T cells binds ^{3}H-dexamethasone significantly (Figure 3A). However, a dramatic decrease in steroid ligand binding activity was observed in HDAC6 knockdown 293T cells (Figure 3A). Although comparable amounts of GR are present in cytosols from both cell types (Figure 3A, Western blot), GR from HDAC6 knockdown cells exhibited approximately a 6-fold reduction in ligand binding activity, relative to control cells. This result demonstrates that GR produced in HDAC6 knockdown cells is defective in ligand binding activity, indicating an Hsp90 chaperone deficiency associated with Hsp90 hyperacetylation.

We further examined the functional status of GR by a transcriptional reporter assay. In control cells, endogenous GR efficiently induced a glucocorticoid-responsive element (GRE)-driven luciferase reporter after addition dexamethasone for 4–6 hr (Figure 3B, control). In contrast, the same dexamethasone treatment only weakly activated the GR reporter in HDAC6 knockdown cells (Figure 3B, knockdown cells). A similar conclusion was reached with prolonged ligand treatment but with a less marked difference (data not shown). Importantly, reintroduction of an siRNA-resistant plasmid expressing wt HDAC6 (Kawaguchi et al., 2003) fully restored GR transcriptional activity in the HDAC6 knockdown cells (Figure 3B, +wt), whereas the catalytically-inactive or BUZ finger-deletion mutant HDAC6 were ineffective (Figure 3B, +cat-mut and +ΔBUZ), consistent with the observations that these mutants do not bind Hsp90 and cannot deacetylate Hsp90 efficiently (Figures 1B and 2A). To further establish that HDAC6 is required for optimal GR activity, we also investigated endogenous GR-target gene induction in response to dexamethasone stimulation. As shown in Figure 3D, a similar defect in endogenous GR-target gene induction by ligand was observed in HDAC6 knockdown cells. Importantly, the observed defect in transcriptional activity in HDAC6 knockdown cells is mirrored by the loss of ligand-induced nuclear accumulation of GR. In control A549 cells, GR becomes almost exclusively localized to the nucleus within 30 min of ligand treatment (Figure 3E, panel ii). In contrast, GR remains in the cytoplasm in the substantial majority (~80%) of HDAC6 knockdown cells after ligand addition (Figure 3E, panel iv). Thus, Hsp90-dependent GR ligand binding, nuclear translocation, and transcriptional activity are all defective in HDAC6 knockdown cells. Together, these results argue that HDAC6-mediated deacetylation is required for Hsp90 chaperone function to activate GR.

Hsp90 Hyperacetylation Is Induced by Dexamethasone and Correlated with the Dissociation of Functional Chaperone-GR Complexes

To begin to understand how acetylation controls Hsp90 activity toward GR function, we first determined whether Hsp90 acetylation is regulated by dexamethasone. As shown in Figure 4A, in control cells, dexamethasone treatment does not have a marked effect. However, in HDAC6 knockdown cells, an increase of acetylation by dexamethasone is evident. These results are consistent with the idea that Hsp90 acetylation is induced upon dexamethasone treatment, and this acetylation is efficiently removed by HDAC6.

We next attempted to identify the molecular basis for the regulation of Hsp90 function via HDAC6-mediated deacetylation. Because the proper folding of GR by Hsp90 depends on the association of Hsp90 with a distinct set of cochaperones into a chaperone complex (reviewed in Neckers, [2002] and Pratt and Toft [2003]), we determined whether Hsp90 acetylation affects Hsp90/cochaperone assembly. We focused on p23 (Johnson and Toft, 1994), a cochaperone that stabilizes the Hsp90-GR complex and is critical for GR ligand binding activity in vitro and in vivo (Dittmar et al., 1997; Morishima et al., 2003). Coimmunoprecipitation assays demonstrate that Hsp90 associates with p23 in control cells (Figure 4B, lane 1). However, Hsp90-p23 interactions are dramatically reduced in HDAC6 knockdown cells (Figure 4B, lane 4). TSA treatment, which induces Hsp90 hyperacetylation, also disrupts Hsp90-p23 interactions (Figure 4B, lane 2). Conversely, TPXb treatment, which does not inhibit HDAC6 activity, has little effect (Figure 4B, lane 3). These results support the hypothesis that Hsp90 acetylation leads to the dissociation of p23 from Hsp90.

As p23 is known to stabilize the Hsp90-GR complex (Dittmar et al., 1997), we next examined whether acetylation affects Hsp90-GR complex formation. Indeed, the Hsp90 and GR interaction is significantly reduced in HDAC6 knockdown cells or by treatment with TSA (Figure 4C, compare lanes 1–3), providing a plausible mechanism for the observed GR defects. These results show that loss of HDAC6 activity leads to Hsp90 hyperacetylation, disassembly of the Hsp90 chaperone complex, and dissociation of the client protein GR.

Discussion

By promoting the activity and stability of many important signaling proteins, the molecular chaperone Hsp90 has recently emerged as a critical modulator in cell signaling and promising target in cancer therapy. Despite

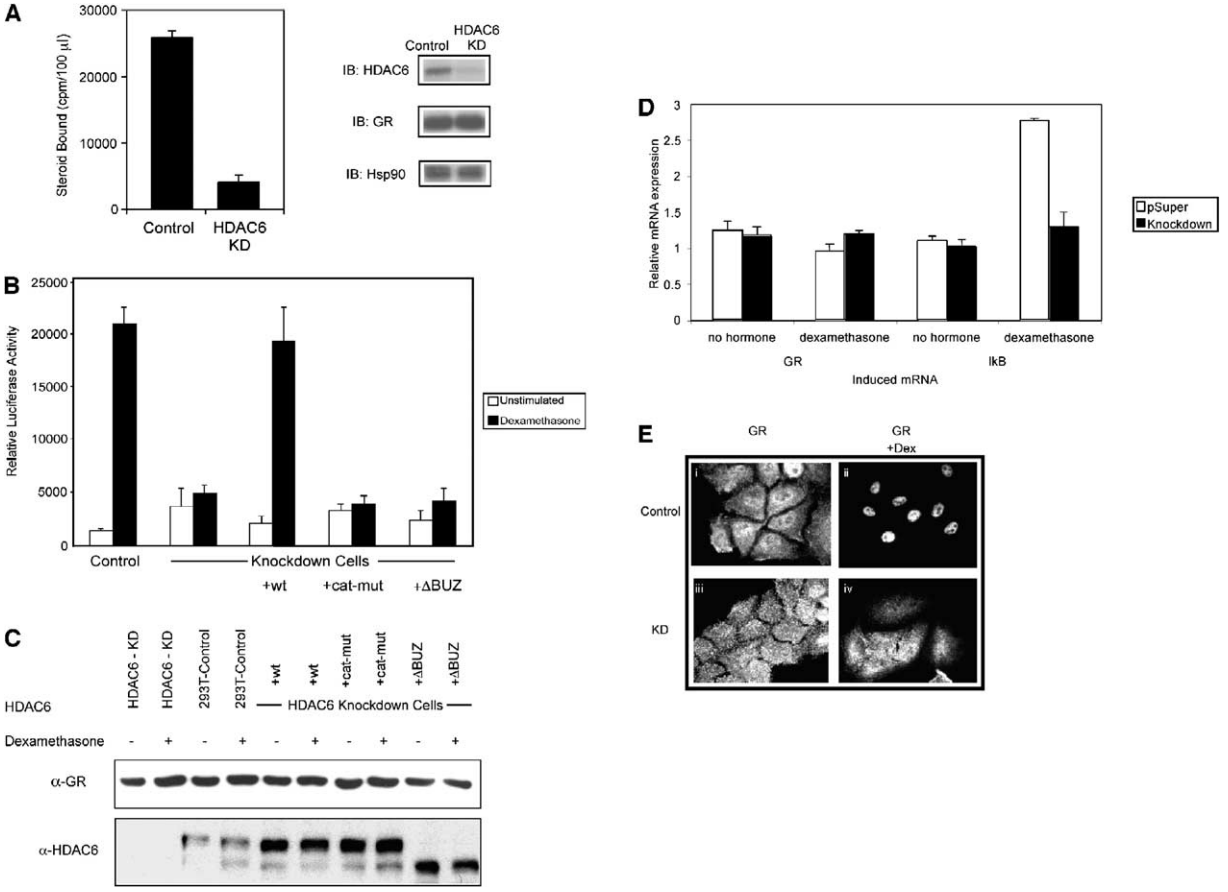

Figure 3. HDAC6 Is Required for GR Ligand Binding, Translocation, and Transcriptional Activity

(A) Cytosols were prepared from control or HDAC6 knockdown 293T cells and subjected to ligand binding assay (see Experimental Procedures). Steroid binding is expressed as cpm of [3H]dexamethasone/100 µl of cell cytosol ± SEM for three experiments with assays performed in triplicate.

(B) Control or HDAC6 knockdown 293T cells were transiently transfected with an MMTV-GRE-luciferase reporter with or without expression plasmids of wt, catalytically inactive (cat-mut), or ΔBUZ mutant HDAC6 as indicated. These plasmids contain silent mutations in the sequences targeted by siRNA for HDAC6. Note that the transfection of a wt-HDAC6 restored GR transcriptional activity in HDAC6 KD cells. Relative luciferase activity was measured after 4 hr treatment with dexamethasone and normalized to internal control (β-galactosidase) $p < 0.005$. Data are the mean of experiments repeated in triplicate.

(C) The protein levels of GR and HDAC6 are determined by immunoblotting with antibodies against GR and HDAC6, respectively.

(D) mRNA levels of GR and IkB at 4 hr after dexamethasone stimulation were determined by quantitative RT-PCR, normalized to the mRNA levels of 36B4. Solid bars represent pSuper stable cell line, and hashed bars represent HDAC6 knockdown stable cells. Data are the mean ± SEM of three biological replicates performed in triplicate. $p < 0.001$ versus dexamethasone-treated cells in the absence of HDAC6 siRNA.

(E) Control and HDAC6 knockdown A549 cells were cultured in hormone free media for 24 hr and then stimulated with dexamethasone for 30 min. The localization of GR was determined by immunostaining with an α-GR antibody. Immunofluorescence microscopy reveals that GR shows a pan-cell staining before dexamethasone treatment in both cell types (i and iii). After dexamethasone treatment, GR efficiently translocates into the nucleus in control (ii), but not in HDAC6 KD cells (iv).

its apparent importance, how Hsp90 activity, its chaperone complex formation, and Hsp90-client protein interactions are regulated is not well understood. In this study, we have identified HDAC6-regulated reversible acetylation as an important mechanism that controls Hsp90 molecular chaperone function.

Hsp90 chaperone complexes stabilize client proteins and, in the case of GR, promote a conformation that allows efficient ligand binding and subsequent nuclear translocation and transcriptional activation. In this report, we demonstrate that GR produced in HDAC6-deficient model cell lines is defective in all three activities (Figure 3), strongly indicating a defect in Hsp90 chaperone function. Importantly, the ligand binding defects of GR in HDAC6 knockdown cells can be rescued by Hsp90 purified from wt cells in vitro (P.J.M.M. and W.B.P., unpublished data). We found that Hsp90-p23 chaperone complex formation (Figure 4B) and the chaperone-client (Hsp90-GR) association (Figure 4C) are both compromised in HDAC6 knockdown cells. The accumulation of hyperacetylated Hsp90 in HDAC6-deficient cells suggests that acetylation negatively regulates Hsp90 function by lowering Hsp90 affinity for the critical cochaperone p23. Thus, stable complexes with client proteins, such as GR, are not formed, resulting in a failure of client protein maturation (Figure 4). This

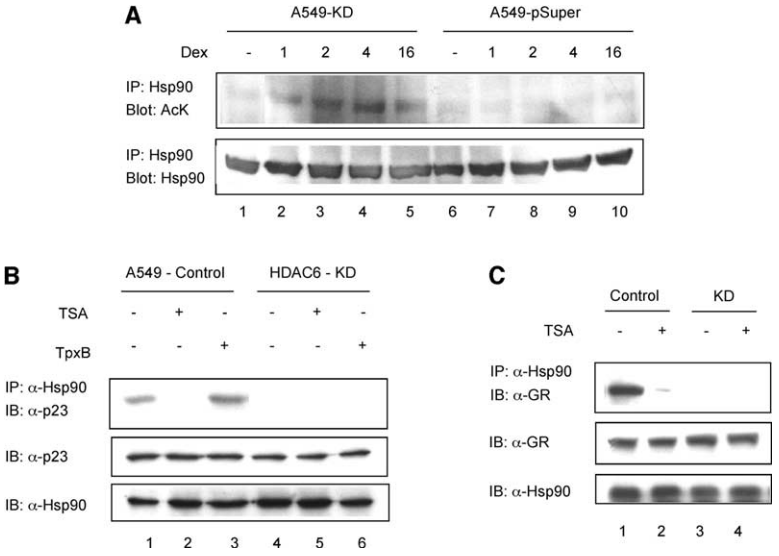

Figure 4. The Acetylation Status of Hsp90 Is Affected by Dexamethasone and Affects Hsp90 Interaction with the Glucocorticoid Receptor and the Cochaperone p23

(A) Control and HDAC6 knockdown A549 cells were cultured in hormone-free media for 24 hr and then stimulated with dexamethasone for the indicated time in hours. Cell lysates were then immunoprecipitated with α-Hsp90 followed by immunoblotting with an α-AcK antibody.

(B) Control A549 cells and HDAC6 siRNA knockdown cells (KD) were left untreated or treated for 4 hr with TSA or TPXb. Lysates were immunoprecipitated with α-Hsp90 antibody followed by immunoblotting with α-p23 antibody.

(C) Lysates from control A549 cells or HDAC6 KD untreated or treated with TSA (4 hr) were immunoprecipitated with α-Hsp90 antibody followed by immunoblotting with α-GR antibody.

model is consistent with two recent reports that several HDAC inhibitors can induce Hsp90 acetylation and the dissociation of oncogenic client proteins from Hsp90 (Nimmanapalli et al., 2003; Xu et al., 2001). Our study strongly suggests that HDAC6 is a primary Hsp90 deacetylase targeted by those inhibitors. However, it is possible that other members of the HDAC family could also regulate Hsp90 acetylation.

Surprisingly, GR remains stable in HDAC6 knockdown cells even though it no longer stably associates with Hsp90 (Figure 3C). This is in contrast to the effect of the Hsp90 inhibitor geldanamycin (GA), which dissociates GR from Hsp90 and induces GR degradation (Whitesell and Cook, 1996). These observations suggest that acetylation and GA, while both interfering with Hsp90 chaperone function toward GR, do not work through identical mechanisms. In fact, Hsp90 client proteins are more resistant to GA-induced degradation in HDAC6 knockdown cells (P.J.M.M. and J.J.K., unpublished data). Understanding how acetylation and GA differentially affect Hsp90 function could provide important insight into the regulation of chaperone-dependent client protein maturation and degradation.

Many questions regarding Hsp90 acetylation remain to be answered. A critical one is how Hsp90 acetylation is regulated. Interestingly, we found that dexamethasone treatment induced transient Hsp90 acetylation, which appears to be efficiently removed by HDAC6 (Figure 4A). Although the physiological significance of dexamethasone-induced Hsp90 acetylation in GR function is not entirely clear, this observation supports the idea that Hsp90 acetylation is regulated by cell signaling events. Acetylation could contribute to the conversion of GR-Hsp90 into a dynamic complex and its dissociation from p23, thereby enabling proper GR function in the nucleus. Acetylation could be involved in Hsp90 nuclear export to the cytoplasm where it can resume its chaperone function. Regardless of the mechanism, because acetylated Hsp90 cannot form a stable complex with GR (Figure 4), the deacetylation of Hsp90 by HDAC6 in the cytoplasm would be required to regenerate and produce Hsp90 competent to chaperone GR and selected client proteins. Consequently, in the absence of HDAC6, acetylated Hsp90 would accumulate leading to defects in GR maturation and function. It is also of interest to note that HDAC6 is a microtubule-associated deacetylase, and it regulates specific microtubule-dependent protein transport (Hubbert et al., 2002; Kawaguchi et al., 2003). Recently, it was proposed that Hsp90 also regulates client protein function by facilitating their intracellular transport to specific subcellular compartments by way of the microtubules (Pratt and Toft, 2003). These observations suggest an intriguing link between Hsp90 acetylation, client protein maturation, and transport. The concentration of HDAC6 at the microtubules also raises an interesting possibility that Hsp90 associated with microtubules might be preferentially subject to regulation by HDAC6. The potential compartmentalization of Hsp90 acetylation and deacetylation could play a role for efficient cell signaling mediated by selected Hsp90 client proteins.

Although the exact mechanism by which acetylation regulates Hsp90 function remains to be established, and there may be additional chaperone components subject to reversible acetylation, our findings support the hypothesis that acetylation acts by modulating Hsp90 chaperone complex formation (Figure 4). Interestingly, hyperacetylated Hsp90 appears to have a lower affinity for ATP (Nimmanapalli et al., 2003). As the assembly of Hsp90-p23 complex requires ATP binding (Schneider et al., 1996), acetylation of Hsp90 could inhibit this functional complex formation, at least in part, by inhibiting ATP binding. Accordingly, HDAC6 would stimulate Hsp90 activity by catalyzing its deacetylation, enhancing ATP binding, and thereby promoting the assembly of functional Hsp90 chaperone complexes. Significantly, a dramatic remodeling of the Hsp90 chaperone complex has been observed upon normal cell transition to a tumor state (Kamal et al., 2003). This observation strongly suggests that the assembly of Hsp90 chaperone complexes is dynamically regulated, and its deregulation might play a key role for normal cells to

undergo oncogenic transformation. It is plausible to speculate that reversible acetylation, controlled by HDAC6 and a yet to be identified molecular chaperone acetyltransferase, may be a physiological modulator that regulates Hsp90 chaperone complex remodeling and activity. Given the importance of Hsp90 for many proteins vital to oncogenic signaling, the discovery of HDAC6 as a critical regulator of Hsp90 acetylation and function could have important therapeutic implications. The identification of Hsp90 as a target for HDAC6 provides further support for broad functions of reversible protein acetylation and HDAC family members in important biological processes beyond histone and chromatin remodeling.

Experimental Procedures

Cell Lines

A549 and NIH-3T3 cell lines overexpressing HDAC6 wt, ΔBUZ, or catalytically inactive mutants were established by using retroviral infection. A549 and 293T cells stably expressing siRNA for HDAC6 were established as described previously (Kawaguchi et al., 2003).

Antibodies

Rabbit polyclonal HDAC6 antibody DU227 was raised against a C-terminal HDAC6 peptide (DVKNAAHQNKFGEDMPHSH) followed by affinity purification. DU184 has been described previously (Hubbert et al., 2002). The production of antibodies for acetylated lysine (Komatsu et al., 2003), Hsp90 (H1090) and p23 (JJ3) has been described (Johnson and Toft, 1994). GR antibody was purchased from Cell Signaling. S-14 antibody recognizing HDAC6 was purchased from Santa Cruz.

Immunoprecipitation and Immunostaining

Cells were lysed as described previously (Hubbert et al., 2002). Hsp90 antibody was preincubated with rabbit-anti-mouse (Jackson Labs) and Protein A Sepharose beads (Roche) for 10 min. The bead/antibody mix was added to 750 μg of whole-cell lysate and incubated at 4°C for 3 hr. Samples were washed four times with 150 mM NETN (Hubbert et al., 2002) and subjected to SDS-PAGE and immunoblotting analysis. Immuolocalization of GR and HDAC6 was described previously (Hubbert et al., 2002).

Ligand Binding Assay

293T cells stably transfected with HDAC6 siRNA or control (pSuper) plasmid were lysed in 1.5 volumes of buffer (10 mM Hepes, [pH 7.35], 1 mM EDTA, and 20 mM Na_2MoO_4) and centrifuged at 100,000 × g. Aliquots (150 μl) of cytosol were incubated overnight at 4°C with 100 nM [3H]dexamethasone ± a 1000-fold excess of nonradioactive dexamethasone. Free steroid was removed with dextran-coated charcoal, and steroid binding is expressed as cpm of [3H]dexamethasone/100 μl of cell cytosol, ± SEM for three experiments with assays performed in triplicate.

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Regulation of the Dynamics of hsp90 Action on the Glucocorticoid Receptor by Acetylation/Deacetylation of the Chaperone*

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It is known that inhibition of histone deacetylases (HDACs) leads to acetylation of the abundant protein chaperone hsp90. In a recent study, we have shown that knockdown of HDAC6 by a specific small interfering RNA leads to hyperacetylation of hsp90 and that the glucocorticoid receptor (GR), an established hsp90 "client" protein, is defective in ligand binding, nuclear translocation, and gene activation in HDAC6-deficient cells (Kovacs, J. J., Murphy, P. J. M., Gaillard, S., Zhao, X., Wu, J-T., Nicchitta, C. V., Yoshida, M., Toft, D. O., Pratt, W. B., and Yao, T-P. (2005) *Mol. Cell* 18, 601–607). Using human embryonic kidney wild-type and HDAC6 (small interfering RNA) knockdown cells transiently expressing the mouse GR, we show here that the intrinsic properties of the receptor protein itself are not affected by HDAC6 knockdown, but the knockdown cytosol has a markedly decreased ability to assemble stable GR·hsp90 heterocomplexes and generate stable steroid binding activity under cell-free conditions. HDAC6 knockdown cytosol has the same ability to carry out dynamic GR·hsp90 heterocomplex assembly as wild-type cytosol. Addition of purified hsp90 to HDAC6 knockdown cytosol restores stable GR·hsp90 heterocomplex assembly to the level of wild-type cytosol. hsp90 from HDAC6 knockdown cytosol has decreased ATP-binding affinity, and it does not assemble stable GR·hsp90 heterocomplexes when it is a component of a purified five-protein assembly system. Incubation of knockdown cell hsp90 with purified HDAC6 converts the hsp90 to wild-type behavior. Thus, acetylation of hsp90 results in dynamic GR·hsp90 heterocomplex assembly/disassembly, and this is manifest in the cell as a ~100-fold shift to the right in the steroid dose response for gene activation.

The hsp90/hsp70-based chaperone machinery regulates a wide variety of proteins involved in cellular signaling through the assembly of client protein·hsp90 heterocomplexes (reviewed in Ref. 1). The chaperones hsp90 and hsp70 play a key role in the balance between maintenance of protein integrity and degradation by the ubiquitin-proteasomal pathway, and inhibition of hsp90 function leads to degradation of hsp90 client proteins (2, 3). Yu *et al.* (4) have reported that treatment of cells with an inhibitor of histone deacetylases (see Refs. 5 and 6 for a review of deacetylase inhibitors) leads to acetylation of hsp90 and depletion of several hsp90 client proteins, including Raf-1, ErbB2, and mutant p53. This suggests that hsp90 function is affected by acetylation/deacetylation.

Histone deacetylase (HDAC)[3] 6 is a cytoplasmic HDAC that is associated with microtubules (7) and has been shown to regulate aggresome formation in response to misfolded protein stress (8). The cystic fibrosis transmembrane conducting regulator (CFTR) is a client protein of hsp90 (9), and a mutant form, CFTR-Δ508, is prone to misfolding and aggresome formation (reviewed in Ref. 10). Misfolded CFTR-Δ508 moves in a dynein-dependent fashion along microtubules to form the perinuclear aggresome (10). HDAC6 is a component of these aggresomes and cells deficient in HDAC6 cannot form aggresomes properly, apparently because of a failure to load polyubiquitinated misfolded protein onto the dynein motor for transport to aggresomes (8). Because ubiquitylated CFTR-Δ508 is coimmunoadsorbed from cytosol with HDAC6 and HDAC6 is coadsorbed with dynein, it was proposed that HDAC6 directly links ubiquitylated CFTR-Δ508 to the motor protein (8).

Other hsp90 client proteins, such as the glucocorticoid receptor (GR) (11) and the tumor suppressor protein p53 (12, 13), have been shown to utilize an hsp90-dependent mechanism for dynein-dependent retrograde movement along microtubules. In this movement system, client protein·hsp90 heterocomplexes formed by the hsp90/hsp70-based chaperone machinery are linked via hsp90-binding immunophilins to the dynein/dynactin motor protein complex (Refs. 11 and 13 and reviewed in Ref. 14). Because HDAC6 could be required for proper hsp90 function in forming these movement complexes rather than acting itself to directly link the client protein to the dynein motor, we have examined the effect of HDAC6 on hsp90 acetylation and function (15).

We showed that HDAC6 binds hsp90 *in vivo* and that purified HDAC6 deacetylates hsp90 *in vitro* (15). Inhibition of histone deacetylases by trichostatin A or knockdown (KD) of HDAC6 by specific small interfering RNA leads to hyperacetylation of hsp90, and hsp90 in cytosol of HDAC6 knockdown cells has much less of the hsp90 cochaperone p23 bound to it than the hsp90 in wild-type cytosol (15). The GR in HDAC6-deficient cells is defective in ligand binding, nuclear translocation, and gene activation, suggesting that HDAC6 is required for hsp90 function *in vivo* (15). Although we proposed that HDAC6 activity is critical for normal GR function, it was not determined that hsp90, rather than the GR itself, p23, or another component of the hsp90/hsp70-based chaperone machinery, was the critical target of HDAC6 action.

In this work, we have examined the role of HDAC6 on GR·hsp90

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[3] The abbreviations used are: HDAC, histone deacetylase; GR, glucocorticoid receptor; hsp, heat shock protein; CFTR, cystic fibrosis transmembrane conducting regulator; KD, knockdown; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

heterocomplex assembly and steroid binding activity *in vitro*. We show that cytosol from HDAC6 knockdown cells has a decreased ability to assemble stable GR·hsp90 heterocomplexes and that the assembly activity can be restored to the level of wild-type cytosol by addition of purified deacetylated hsp90. The stable heterocomplex assembly activity of hsp90 purified from HDAC6 knockdown cytosol can be functionally restored by incubating it with purified HDAC6. Interestingly, despite its deficiency in stable heterocomplex assembly, the acetylated hsp90 of HDAC6 knockdown cells is still able to carry out dynamic assembly of GR·hsp90 heterocomplexes. The acetylated hsp90 has a lower affinity for ATP than the wild-type hsp90, and the dynamic GR·hsp90 heterocomplex assembly/disassembly that occurs in HDAC6 knockdown cells is manifest as a ~100-fold shift to the right *versus* wild-type cells in the dose response of dexamethasone for transcriptional activation. Together with our previous report (15), our observations prove that hsp90 function is regulated through acetylation/deacetylation.

EXPERIMENTAL PROCEDURES

Materials

Untreated rabbit reticulocyte lysate was purchased from Green Hectares (Oregon, WI). [6,7-^{3}H]Dexamethasone (40 Ci/mmol), [*ring*-3,5-^{3}H]chloramphenicol (38 Ci/mmol), and ^{125}I-conjugated goat anti-mouse and goat anti-rabbit IgGs were obtained from PerkinElmer Life Sciences. Protein A-Sepharose, non-radioactive dexamethasone, trichostatin A, goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies, and M2 monoclonal anti-FLAG IgG were from Sigma. Dulbecco's modified Eagle's medium was from Bio-Whittaker (Walkersville, MD). The BuGR2 monoclonal IgG used to immunoblot the mouse GR and the rabbit polyclonal antibody used to immunoblot human GR were from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG used to immunoblot hsp90 was from StressGen Biotechnologies (Victoria, BC, Canada). The JJ3 monoclonal IgG used to immunoblot p23 was a gift from Dr. David Toft (Mayo Clinic, Rochester, MN). The FiGR monoclonal IgG used to immunoadsorb the mouse GR was generously provided by Dr. Jack Bodwell (Dartmouth Medical School, Lebanon, NH), and the 8D3 monoclonal IgM used to immunoadsorb hsp90 was kindly provided by Dr. Gary Perdew (Pennsylvania State University, University Park, PA). The pSV2Wrec plasmid encoding full-length mouse GR and the mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter plasmid were kindly provided by Dr. Edwin Sanchez (Medical College of Ohio, Toledo, OH). 293T human embryonic kidney cells stably expressing a pSuper control small interfering RNA (293T-wt) or HDAC6 small interfering RNA (293T-HDAC6KD) were described previously (8). The expression plasmid pcDNA3-FLAG-tagged HDAC6 and rabbit antisera used to immunoblot HDAC6 (α-HDAC6) and acetylated lysine (α-AcK) were generated in the Yao laboratory and have been described (7, 8).

Methods

Cell Culture and Cytosol Preparation—293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. Cells were harvested by scraping into Hanks' buffered saline solution and centrifugation. Cell pellets were washed in Hanks' buffered saline solution, resuspended in 1.5 volumes of HEM buffer (10 mM NaOH-Hepes, 1 mM EDTA, and 20 mM sodium molybdate, pH 7.4) with 1 μM trichostatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of Complete-Mini protease inhibitor mixture (Roche Applied Science) per 3 ml buffer, and ruptured by Dounce homogenization. The lysate was then centrifuged at 100,000 × *g* for 30 min, and the supernatant, referred to as "cytosol," was collected, aliquotted, flash-frozen, and stored at −70 °C. Mouse GR was expressed in Sf9 cells, and cytosol was prepared as described previously (16).

Transient Transfection of Mouse GR and MMTV-CAT Reporter—293T cells were grown as monolayer cultures in 162-cm^2 culture flasks to ~50% confluency, washed, and incubated with 5 ml of serum-free medium containing 25 μg of plasmid DNA and 75 μl of TransFast transfection reagent (Promega). After 1 h, 10 ml of Dulbecco's modified Eagle's medium with 10% bovine calf serum was added, and the incubations were continued for 48 h. For transfection of MMTV-CAT reporter, wild-type and knockdown cells were grown as monolayer cultures in 35-mm culture wells to ~50% confluency, washed, and incubated for 1 h with 1 ml of serum-free medium containing 5 μg of plasmid DNA and 15 μl of TransFast transfection reagent. The transfection medium was replaced with regular medium, and the cells were incubated for 48 h. During the incubation, cells were treated for 20 h with various concentrations of dexamethasone.

Immunoadsorption of GR—Receptors were immunoadsorbed from aliquots of 50 μl (for measuring steroid binding) or 100 μl (for Western blotting) of Sf9 cell cytosol by rotation for 2 h at 4 °C with 18 μl of protein A-Sepharose precoupled to 10 μl of FiGR ascites suspended in 200 μl of TEG (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol). Immunoadsorbed GR was stripped of endogenously associated hsp90 by incubating the immunopellet for an additional 2 h at 4 °C with 350 μl of 0.5 M NaCl in TEG buffer. The pellets were then washed once with 1 ml of TEG buffer followed by a second wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4).

GR·hsp90 Heterocomplex Reconstitution—Immunopellets containing GR stripped of chaperones were incubated with 50 μl of reticulocyte lysate or 293T cell cytosol and 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase). For heterocomplex reconstitution with purified proteins, immunopellets containing stripped GR were incubated with 15 μg of ATP-agarose-purified knockdown or wild-type HEK 293 hsp90, 15 μg of purified rabbit hsp70, 0.6 μg of purified human Hop, 6 μg of purified human p23, 0.125 μg of purified YDJ-1 adjusted to 55 μl with HKD buffer (10 mM Hepes, pH 7.4, 100 mM KCl, 5 mM dithiothreitol) containing 20 mM sodium molybdate and 5 μl of the ATP-regenerating system. The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG with 20 mM sodium molybdate) and assayed for steroid binding capacity and for GR-associated hsp90. In the experiments shown in Fig. 7, the five-protein mixture containing purified knockdown hsp90 was incubated for 5 min at 30 °C with an α-FLAG immune pellet prepared from cytosols of control or FLAG-tagged HDAC6-expressing cells prior to addition of the mixture to stripped GR immune pellets for heterocomplex reconstitution for 20 min at 30 °C.

Assay of Steroid Binding Capacity—For cytosols to be assayed for steroid binding, a 50-μl aliquot of cytosol was incubated overnight at 4 °C in 50 μl of HEM buffer plus 50 nM [^{3}H]dexamethasone ± a 1,000-fold excess of non-radioactive dexamethasone. Samples were mixed with dextran-coated charcoal, centrifuged, and counted by liquid scintillation spectrometry. The steroid binding is expressed as counts/min of [^{3}H]dexamethasone bound/100 μl of cytosol.

Washed immune pellets to be assayed for steroid binding to stable GR·hsp90 heterocomplexes were incubated overnight at 4 °C in 50 μl of HEM buffer plus 50 nM [^{3}H]dexamethasone. Samples were then washed three times with 1 ml of TEGM buffer and counted by liquid scintillation spectrometry. For assay of steroid binding under dynamic

GR·hsp90 assembly conditions, 50 nM [^{3}H]dexamethasone was present during the assembly incubation at 30 °C, and pellets were then washed and counted. In both cases, the steroid binding is expressed as counts/min of [^{3}H]dexamethasone bound/FiGR immunopellet prepared from 100 μl of Sf9 cell cytosol.

Gel Electrophoresis and Western Blotting—Immune pellets were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 0.25 μg/ml BuGR2 for GR, 1 μg/ml AC88 for hsp90, 1 μg/ml JJ3 for p23, 0.1% α-AcK, or 0.1% α-HDAC6. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated or horseradish peroxidase-conjugated counter-antibody to visualize the immunoreactive bands.

Protein Purification—hsp70, Hop, YDJ-1 (the yeast homolog of hsp40), and p23 were purified as described by Kanelakis and Pratt (17). When hsp90 was purified from HDAC6 knockdown cells by our usual three-step procedure (17), it was deacetylated and functionally identical to purified wild-type hsp90 in supporting stable GR·hsp90 heterocomplex assembly in the five-protein assembly system. Because hsp90 binds to ATP-agarose when the salt concentration of the application buffer is low and can then be eluted with a salt gradient, we used a single-step procedure of ATP-agarose chromatography both to partially purify hsp90 and to compare the relative ATP-binding properties of hsp90 from knockdown and wild-type cells. This procedure rapidly separates hsp90 from deacetylating activity and yields acetylated hsp90 from knockdown cytosol that does not support stable GR·hsp90 heterocomplex assembly (Fig. 6). For ATP affinity chromatography, 2.0 ml of cytosol prepared in HEM buffer was applied to a 50-ml column of ATP-agarose, the column was washed with 100 ml of HE buffer (10 mM Hepes, pH 7.4, 2 mM EDTA), and the column was then eluted with a 125-ml gradient of (0–500 mM) KCl in HE buffer. hsp90 was eluted with the KCl gradient, and the matrix was subsequently cleared of hsp70 and other high affinity ATP-binding proteins by elution with 5 mM ATP. The hsp90-containing fractions were identified by Western blotting, pooled, and contracted to 200–250 μ by Amicon filtration. It is important to freeze the preparation in multiple small aliquots and to unfreeze them only once.

GR-mediated Transcriptional Activation—Dexamethasone-induced CAT gene expression was assayed by measuring CAT enzymatic activity in wild-type and knockdown cell cytosol, using a modified version of the CAT assay described in Kwok et al. (18). Cells transfected with MMTV-CAT reporter and treated for 20 h with various concentrations of dexamethasone were washed, harvested, resuspended in potassium phosphate buffer (100 mM potassium phosphate, 1 mM dithiothreitol, pH 7.8), and ruptured by exposing the cell suspensions to three freeze-thaw cycles. Cell suspensions were centrifuged at 18,000 × *g* for 10 min, and the protein concentration of the supernatants was measured by Bradford assay. Aliquots of the supernatants containing 10 μg of total protein were incubated for 15 min at 70 °C in 150 mM Tris-HCl buffer, pH 7.4. The aliquots were added to a CAT reaction mixture (50 nM purified [^{3}H]chloramphenicol, 150 mM Tris-HCl, pH 7.4, 0.25 mM butyryl-CoA) and incubated for 2 h at 37 °C. An organic phase mixture consisting of 2 parts pristane and 1 part mixed xylenes was added, and the samples were thoroughly vortexed. The reaction mixture was centrifuged at 20,000 × *g* for 10 min, and 150 μl of the organic phase was counted by liquid scintillation spectrometry.

RESULTS

hsp90 Binding to GR and Steroid Binding Activity Are Decreased in HDAC6 Knockdown Cells—FK228, an inhibitor of multiple histone deacetylases, has been reported to deplete cells of several hsp90 client proteins (e.g. p53, ErbB2, Raf-1) (4). However, selective knockdown of HDAC6 in HEK 293T cells results in decreased glucocorticoid binding activity without any decrease in the level of endogenous human GR (Fig. 1*A*). To further examine the effect of HDAC6 knockdown, we wanted to immunoadsorb the GR and directly examine its activity in formation of GR·hsp90 heterocomplexes. Because we were unable to find an immunoadsorbing antibody against the human GR, we transiently expressed the mouse (m)GR in HEK cells. Again, we found decreased steroid binding activity in HDAC6 knockdown cells without any decrease in the level of expressed mGR (Fig. 1*B*). mGR was immunoadsorbed from both wild-type and HDAC6 knockdown cells, and the immune pellets were immunoblotted to detect coadsorbed hsp90 and p23. As shown in Fig. 1*C*, there is very little hsp90 or p23 in mGR immune pellets from knockdown cells compared with wild-type cells.

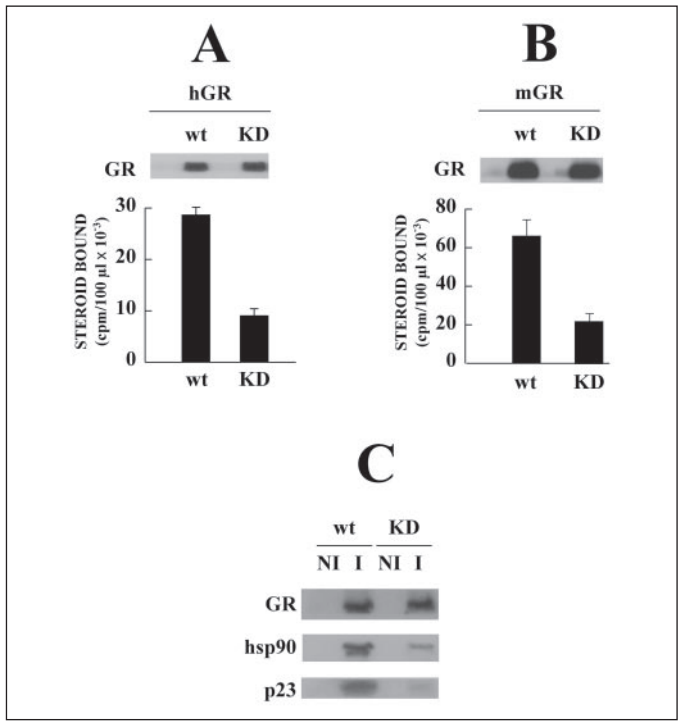

FIGURE 1. **Both steroid binding activity and GR·hsp90 heterocomplex assembly are deficient in HDAC6 knockdown cells.** *A*, steroid binding to human GR in HEK cells. Cytosol prepared from 293T wild-type (*wt*) and 293T-HDAC6KD (*KD*) cells was Western blotted for human GR (*hGR*) expression or assayed for steroid binding activity. *B*, steroid binding to mouse GR in HEK cells. Cytosol prepared from 293T cells transiently transfected with mouse GR (*mGR*) was Western blotted for mGR expression or assayed for steroid binding activity. The steroid binding values represent the mean ± S.E. from three experiments. *C*, effect of HDAC6 knockdown on GR·hsp90 heterocomplex assembly. mGR was immunoadsorbed from 293T cell cytosol prepared as in *B* with anti-GR (*I*) or a nonimmune IgG (*NI*), and the immune pellets were washed and Western blotted for mGR, hsp90, and p23.

The GR in HDAC6 Knockdown Cells Has Normal Ability to Form Complexes with hsp90—The cochaperone p23 stabilizes GR·hsp90 complexes, both in cell-free assembly (19) and in vivo (20). Thus, it is possible that acetylation of p23 or of the GR itself could account for decreased steroid binding activity and decreased recovery of GR·hsp90 heterocomplexes from HDAC6 knockdown cells. However, we did not detect any acetylation of either the mGR or p23 immunoadsorbed from knockdown cells under conditions where acetylation of knockdown hsp90 could be visualized (Fig. 2*A*). To determine whether the mGR from HDAC6 knockdown cells could form stable GR·hsp90 complexes, the mGR was incubated with rabbit reticulocyte lysate. As shown in Fig. 2*B*, the mGR from knockdown cells had the same ability to form mGR·rabbit hsp90 heterocomplexes with the same steroid binding

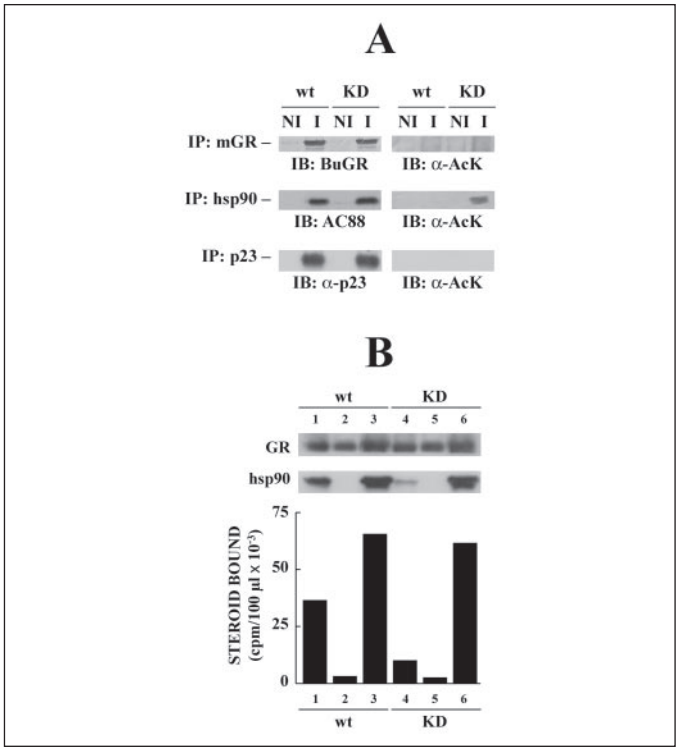

FIGURE 2. **The GR itself is not affected by HDAC6 knockdown.** *A*, neither GR nor p23 is acetylated in HDAC6 knockdown cells. Aliquots of wild-type (*wt*) or HDAC6KD (*KD*) cytosol were immunoadsorbed with antibody against GR, p23 or hsp90 (*I*), or nonimmune IgG (*NI*), and the washed immune pellets were immunoblotted (*IB*) with α-GR (*BuGR*), α-p23, α-hsp90 (*AC88*), or α-acetylated lysine (*α-AcK*) antibody. *B*, GRs in wild-type and knockdown cells are activated equivalently by reticulocyte lysate. Mouse GR·human hsp90 heterocomplexes were immunoadsorbed from cell cytosols of 293T wild-type (*wt*) and 293T-HDAC6KD (*KD*) cells transiently transfected with mGR. mGR was salt-stripped of human hsp90 and associated proteins and incubated for 20 min at 30 °C with reticulocyte lysate in the presence of an ATP-generating system. Immunopellets were washed and Western blotted for mGR and hsp90. Duplicate pellets were washed, incubated overnight with [^{3}H]dexamethasone, and assayed for steroid binding activity. *Lanes 1* and *4*, native GR·hsp90 complexes; *lanes 2* and *5*, stripped GR; *lanes 3* and *6*, stripped GR incubated with reticulocyte lysate.

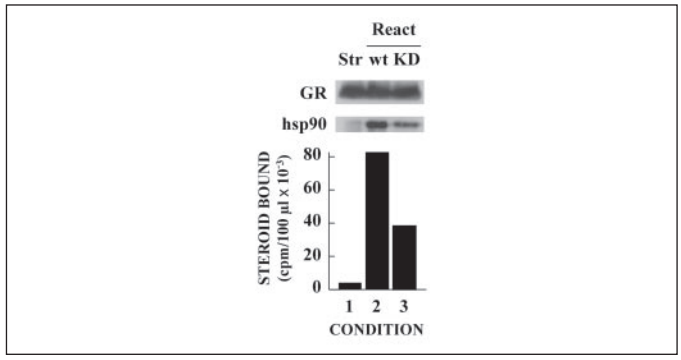

FIGURE 3. **Cytosol from HDAC6 knockdown cells is deficient at assembly of GR·hsp90 heterocomplexes.** mGR overexpressed in Sf9 cells was immunoadsorbed from cytosol, washed, and stripped with 0.5 M NaCl (*Str*, condition 1). Immunopellets were incubated for 20 min at 30 °C with 50 µl of cell cytosol prepared from 293T wild-type (*wt*, condition 2) or 293T-HDAC6KD (*KD*, condition 3) cells in the presence of an ATP-generating system. Pellets were washed and Western blotted for GR and hsp90. Duplicate pellets were washed, incubated overnight with [^{3}H]dexamethasone, and assayed for steroid binding activity.

activity as mGR from wild-type cells. Thus, the mGR from HDAC6 knockdown cells appears to be intrinsically normal and competent to become a functional receptor in the presence of wild-type hsp90 chaperone machinery.

HDAC6 Knockdown Cytosol Is Deficient at Stable GR·hsp90 Heterocomplex Assembly—To determine whether HDAC6 knockdown cells were deficient at GR·hsp90 heterocomplex assembly, baculovirus-expressed mGR was immunoadsorbed from Sf9 cytosol, stripped of insect chaperones, and incubated with cytosols prepared from wild-type and knockdown cells. It can be seen in Fig. 3 that cytosol from HDAC6 knockdown cells has a reduced ability to form GR·hsp90 heterocomplexes and generate steroid binding activity.

In all cases where client proteins form heterocomplexes with hsp90 that are stable enough to survive immunoadsorption and washing, inhibition of hsp90 function (e.g. by geldanamycin) leads to degradation via the ubiquitylation/proteasome pathway (3). This is the case for the GR (21), and it is surprising that the level of GR in HDAC6 knockdown cells is the same as that in wild-type cells (Fig. 1), despite the reduced ability of knockdown cells to form GR·hsp90 heterocomplexes. However, in some cases, hsp90 client proteins engage in a very dynamic cycle of heterocomplex assembly/disassembly, with disassembly being so rapid that no, or only trace amounts of, client protein·hsp90 heterocomplexes are observed with biochemical techniques. This is the case with neuronal nitric-oxide synthase, for example, which associates with hsp90 in a very dynamic manner that is sort of a "hit-and-run" mode of hsp90 regulation (22). However, such a dynamic cycle of heterocomplex assembly/disassembly nevertheless stabilizes neuronal nitric-oxide synthase to proteasomal degradation (23).

The GR undergoes a similar dynamic cycle of hsp90 heterocomplex assembly/disassembly *in vitro* when p23 is omitted from the purified assembly system (19). Such a dynamic assembly cycle can be detected by having radiolabeled dexamethasone present during the assembly incubation (24). The [^{3}H]dexamethasone binds to the receptor as GR·hsp90 complexes are formed, thus steroid binding constitutes evidence that the chaperone machinery has carried out hsp90-dependent opening of the steroid binding cleft. In Fig. 4, replicate GR immune pellets were incubated with cytosols from wild-type and HDAC6 knockdown 293T cells in the absence of dexamethasone and then washed and incubated with [^{3}H]dexamethasone to detect stable heterocomplex assembly (Fig. 4*A*), or they were incubated with cytosols in the presence of [^{3}H]dexamethasone to detect dynamic heterocomplex assembly (Fig. 4*B*). Although the HDAC6 knockdown cytosol is deficient at stable GR·hsp90 heterocomplex assembly, it has the same activity as wild-type cytosol at dynamic heterocomplex assembly.

Purified Rabbit hsp90 Restores Stable Heterocomplex Assembly of HDAC6 Knockdown Cytosol to the Level of Wild-type Cytosol—To determine whether the decreased stable heterocomplex assembly activity of HDAC6 knockdown cytosol was because of altered function of hsp90, purified rabbit hsp90 was added to knockdown cytosol and GR·hsp90 heterocomplex assembly activity and steroid binding activity were assayed. As shown in Fig. 5A, addition of purified hsp90 brings stable heterocomplex assembly activity and steroid binding activity up to the levels of wild-type cytosol. This suggests that components of the assembly machinery other than hsp90 are not affected by HDAC6 knockdown.

We have recently reported that hsp90 immunoadsorbed from knockdown cells has much less p23 bound to it (15). Thus, it is possible that acetylation of hsp90 reduces its p23-binding affinity and that increasing the concentration of p23 could overcome this deficiency. We have shown previously that the stoichiometry of p23 to hsp90 in reticulocyte lysate is ~1:9 and that p23 is the limiting component of the hsp90/hsp70-based chaperone system in lysate (20). When purified p23 is added to reticulocyte lysate to achieve approximate stoichiometric equivalence with hsp90, there is an increase in stable GR·hsp90 heterocomplex recovery and steroid binding activity (20). A similar increase in

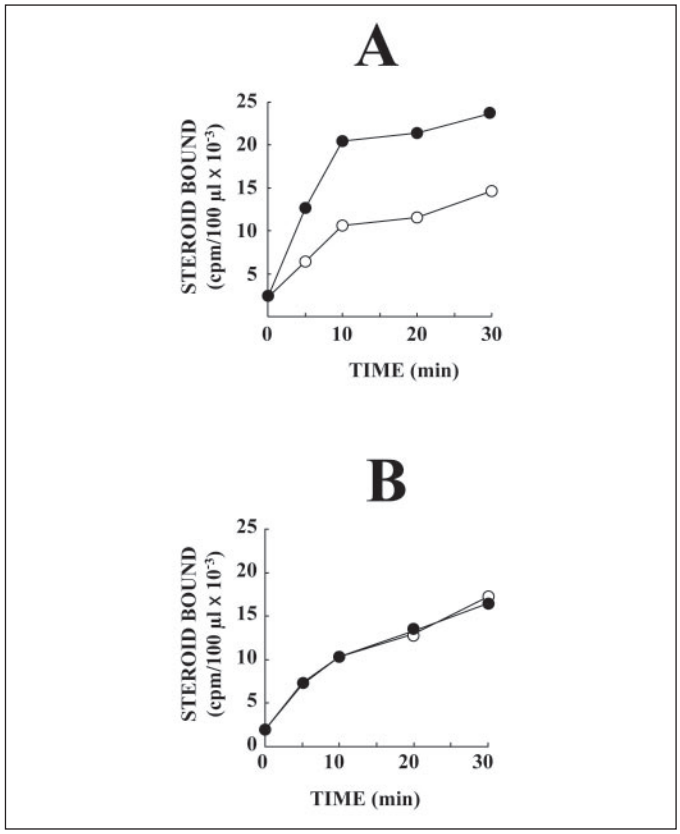

FIGURE 4. **Although deficient at stable GR·hsp90 heterocomplex assembly, HDAC6 knockdown cytosol has the same ability to carry out dynamic assembly as wild-type cytosol.** *A*, stable heterocomplex assembly. mGR overexpressed in Sf9 cells was immunoadsorbed from cell cytosol, washed, and stripped with 0.5 M NaCl. Immunopellets were incubated for 0–30 min at 30 °C with 50 µl of cell cytosol prepared from 293T wild-type (●) or 293T-HDAC6KD (○) cells in the presence of an ATP-generating system. Pellets were washed free of unbound protein, incubated overnight with [^{3}H]dexamethasone, and steroid binding activity was assayed. *B*, dynamic heterocomplex assembly. Stripped immunopellets prepared as in *A* were incubated for 0–30 min at 30 °C with wild-type cytosol (●) or HDAC6 KD cytosol (○) in the presence of an ATP-generating system and 50 nM [^{3}H]dexamethasone. Pellets were washed free of unbound protein, and steroid binding was assayed.

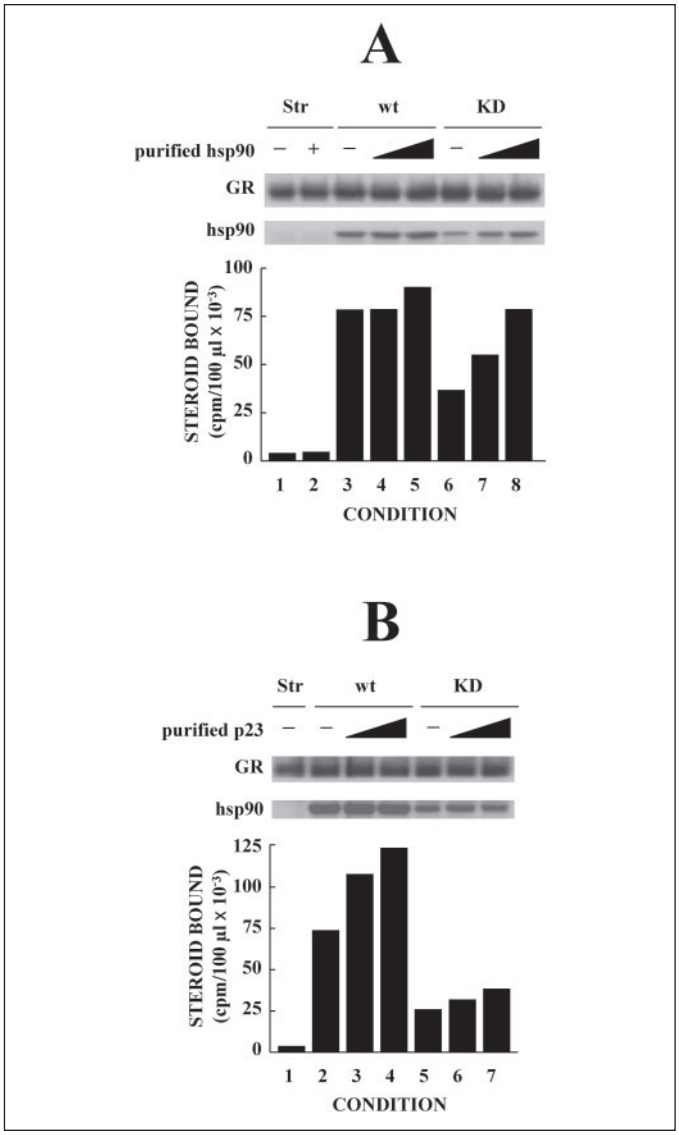

FIGURE 5. **Addition of purified hsp90 restores the steroid binding activity and GR·hsp90 heterocomplex assembly of HDAC6 knockdown cytosol to the level of wild-type cytosol.** *A*, purified hsp90 restores the heterocomplex assembly activity of HDAC6KD cytosol. Stripped mGR (*Str*) immunopellets were incubated for 20 min at 30 °C with 40 µl of cytosol prepared from 293T-wild-type (*wt*) or 293T-HDAC6KD (*KD*) cells in the presence of increasing concentrations of purified hsp90 and an ATP-generating system. Pellets were washed and Western blotted for mGR and hsp90. Duplicate samples were washed free of unbound protein, incubated overnight with [^{3}H]dexamethasone, and assayed for steroid binding activity. Amounts of hsp90 added were: 0 µg (*lanes 1*, *3*, and *6*), 7.5 µg (*lanes 4* and *7*), and 15 µg (*lanes 2*, *5*, and *8*). *B*, p23 does not restore heterocomplex assembly. Stripped immunopellets were incubated as in *A* in the presence of increasing concentrations of purified p23 and an ATP-generating system. Amounts of p23 added were: 0 µg (*lanes 1*, *2*, and *5*), 4 µg (*lanes 3* and *6*), and 8 µg (*lanes 4* and *7*).

stable assembly is seen when purified p23 is added to wild-type 293T cytosol (Fig. 5*B*). Addition of purified p23 to HDAC6 knockdown cytosol yields the same percentage increase in stable assembly, but it does not alter the deficiency in assembly with respect to wild-type cytosol (Fig. 5*B*). Thus, it seems unlikely that acetylation of hsp90 just reduces its affinity for p23, and it is likely that acetylation makes hsp90 unable to respond to p23 at all.

Purified hsp90 from HDAC6 Knockdown Cells Has Decreased ATP-binding Affinity—Cytosols prepared from HDAC6 knockdown 293T cells are deficient at stable GR·hsp90 heterocomplex assembly, but they nevertheless have 20–50% of the stable assembly activity of cytosols from wild-type cells (Figs. 3–5). The ability to form some stable heterocomplexes suggests that there is a mixture of acetylated and deacetylated hsp90 in knockdown cytosol. We purified hsp90 from knockdown cytosol using our usual three-step protocol involving sequential chromatography on DEAE-cellulose, hydroxyapatite, and ATP-agarose (17). The purified hsp90 was then assayed for GR·hsp90 heterocomplex assembly activity in a five-protein mixture containing purified rabbit hsp70, purified human Hop, purified human p23, and purified YDJ-1, the yeast homolog of hsp40 (17). Unexpectedly, the purified HDAC6 knockdown cell hsp90 had the same activity at stable GR·hsp90 heterocomplex assembly as hsp90 purified from wild-type 293T cells (data not shown). This suggested that the knockdown cell hsp90 was deacetylated during its purification, and partial deacetylation probably also occurred when knockdown cytosol was incubated at 30 °C during GR·hsp90 heterocomplex assembly.

With the goal of minimizing deacetylation, we wanted a rapid, single-step isolation of hsp90 from the bulk of 239T cytosol proteins. Our experience with purification of rabbit hsp90 is that the chaperone is retained by ATP-agarose under low salt conditions but appears in the dropthrough fraction if it is applied to the column in buffer containing 500 mM KCl (17). Thus, we applied HDAC6 knockdown or wild-type 293T cytosol prepared in low salt buffer to a column of ATP-agarose and eluted with a gradient of 0–500 mM KCl. The hsp90-containing

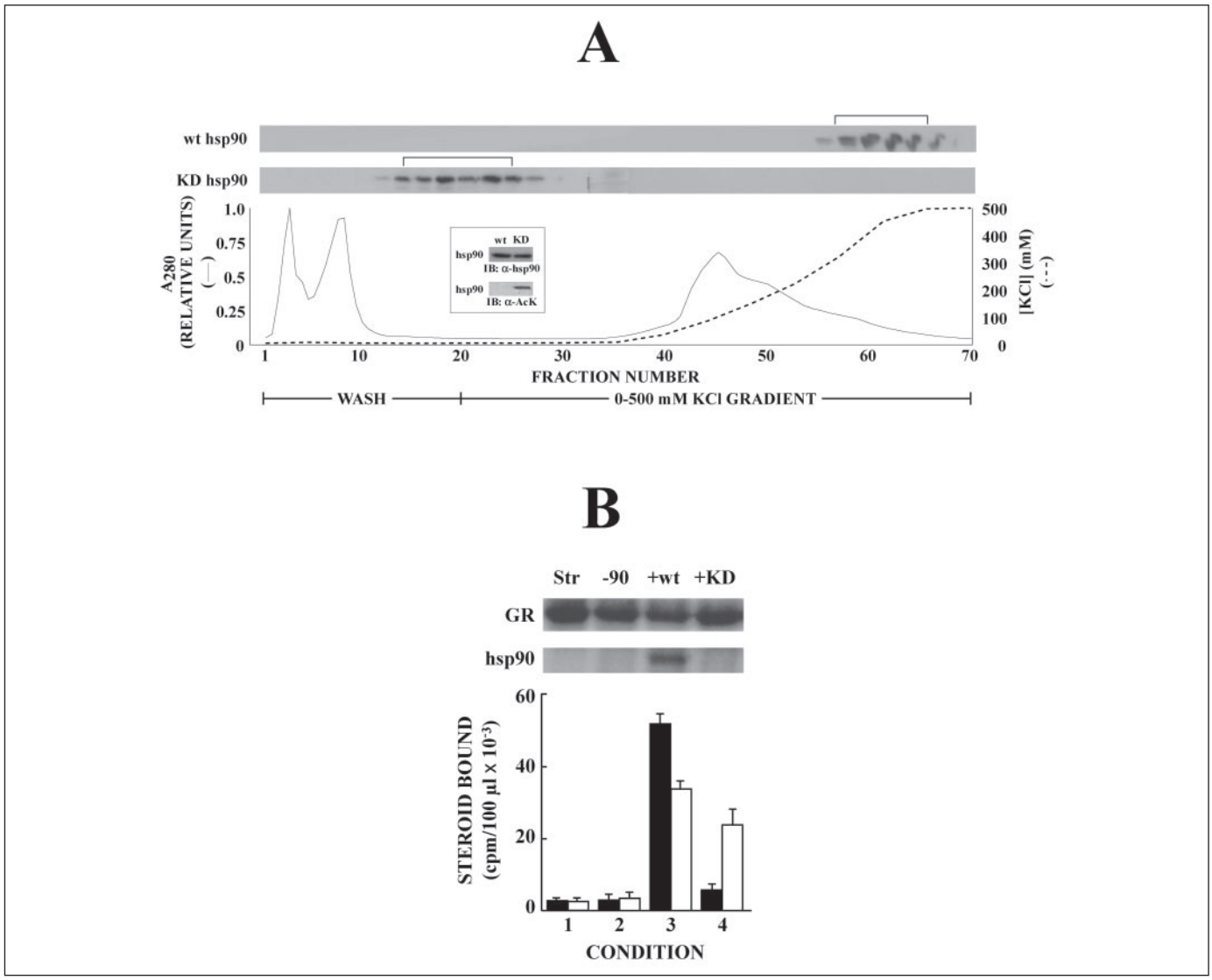

FIGURE 6. **hsp90 from HDAC6 knockdown cells has very low ATP-binding affinity compared with wild-type hsp90.** *A*, hsp90 elution from ATP-agarose. Wild-type (*wt*) or HDAC6KD (*KD*) cytosol was chromatographed on a column of ATP-agarose and hsp90 was identified by immunoblotting (*IB*). The hsp90-containing fractions indicated by brackets were pooled and contracted, and the *inset* presents immunoblots of aliquots of each hsp90 pool to show that the knockdown cell hsp90 is acetylated. *B*, activity of ATP-agarose-purified hsp90 in GR·hsp90 assembly. Wild-type and KD hsp90 prepared in *A* were tested for GR·hsp90 assembly in a mixture containing purified hsp70, Hop, YDJ-1 and p23. Conditions are: stripped GR immunopellet incubated with buffer (*lane 1*), with the four protein mixture without hsp90 (*lane 2*), with mixture and wild-type hsp90 (*lane 3*), with mixture and knockdown hsp90 (*lane 4*). Steroid binding was assayed under conditions of stable assembly (*solid bars*) or dynamic assembly (*open bars*), and binding values represent the mean ± S.E. from three experiments.

fractions identified by immunoblotting were pooled, contracted, and tested for both stable and dynamic GR·hsp90 assembly in the purified five-protein system. As shown in Fig. 6*A*, the HDAC6 knockdown cell hsp90 elutes from ATP-agarose at a low salt concentration and hsp90 from the wild-type cell elutes at high salt. This suggests that the acetylated hsp90 in knockdown cells has a lower ATP-binding affinity than the deacetylated hsp90 in wild-type cells. The hsp90 purified from HDAC6 knockdown cells has no stable GR·hsp90 heterocomplex assembly activity in the purified five-protein system (Fig. 6*B*, *solid bar*), but it retains dynamic assembly activity (Fig. 6*B*, *open bar*).

Incubation with HDAC6 Restores Stable GR·hsp90 Heterocomplex Assembly Activity to Knockdown hsp90—We have shown previously that immunopurified FLAG-HDAC6 deacetylates hsp90 whereas a catalytically dead HDAC6 mutant does not (15). In the experiments in Fig. 7, the five-protein assembly mixture containing purified HDAC6 knockdown cell hsp90 was incubated with immunopurified FLAG-HDAC6, and stable GR·hsp90 heterocomplex assembly was assayed. It can be seen that the stable heterocomplex assembly activity of purified knockdown cell hsp90 (Fig. 7, *lane 3*) is restored to the level of purified wild-type hsp90 (*lane 2*) by incubation with immunopurified FLAG-HDAC6 (*lane 4*). Incubation of wild-type hsp90 with FLAG-HDAC6 does not affect its ability to generate steroid binding (data not shown).

The Dexamethasone Dose-response Curve Is Shifted to the Right ~100-Fold in HDAC6 Knockdown Cells—The GR contains a short 7-amino-acid segment at the N terminus of the ligand binding domain that is required for hsp90 binding and steroid binding activity (25). Mutations of three amino acids in this segment of the rat GR to alanine (P548A/V551A/S552A) yields a triple mutant GR that engages in dynamic GR·hsp90 heterocomplex assembly/disassembly *in vivo* (26). The dose-response curve for dexamethasone-dependent gene transactivation is shifted ~300-fold to the right in cells expressing the triple mutant GR compared with the wild-type GR (26). Because GR·hsp90 heterocomplex assembly/disassembly is similarly dynamic in HDAC6 knockdown 293T cells, we asked whether they had the same phenotype

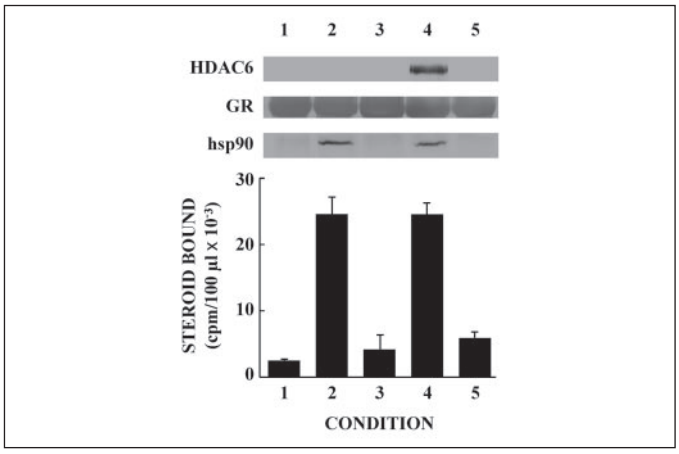

FIGURE 7. **Incubation of purified knockdown hsp90 with HDAC6 restores its stable GR·hsp90 heterocomplex assembly activity.** The five-protein assembly mixture containing hsp90 purified from HDAC6 knockdown cytosol was incubated with immune pellets prepared with α-FLAG from cytosol of nonexpressing or FLAG-tagged HDAC6-expressing cells for 5 min at 30 °C prior to addition to stripped GR immune pellets for stable GR·hsp90 heterocomplex assembly. Conditions are: stripped GR immunopellet incubated with the four-protein mixture without hsp90 (*lane 1*), with mixture and wild-type hsp90 (*lane 2*), with mixture and knockdown hsp90 (*lane 3*), with mixture containing knockdown hsp90 incubated with α-FLAG immune pellet of FLAG-HDAC6-expressing cytosol (*lane 4*), with mixture containing knockdown hsp90 incubated with α-FLAG immune pellet of nonexpressing cytosol (*lane 5*). The steroid binding values represent the mean ± S.E. from three experiments.

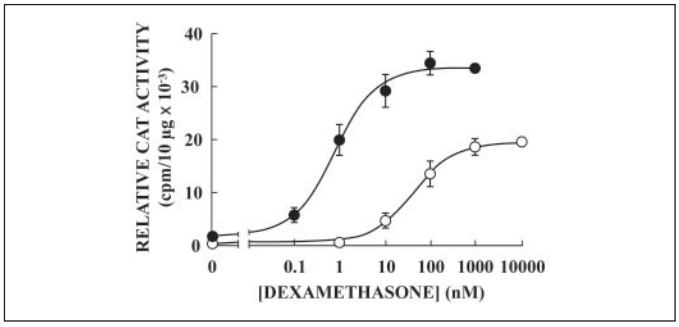

FIGURE 8. **The dexamethasone dose response is shifted to the right in HDAC6 knockdown cells *versus* wild-type cells.** 293T wild-type cells (*closed circles*) and HDAC6 knockdown cells (*open circles*) were transiently transfected with MMTV-CAT reporter plasmid as described under "Experimental Procedures" and treated for 20 h with various concentrations of dexamethasone. The data represent the mean ± S.E. for three experiments expressed as relative CAT activity. The curves were drawn using a nonlinear regression fit provided from Prism software.

with regard to the dose response for dexamethasone. Wild-type or HDAC6 knockdown 293T cells transiently transfected with an MMTV-CAT reporter plasmid were treated for 20 h with various concentrations of dexamethasone prior to assay of CAT activity. As shown in Fig. 8, the dose response for dexamethasone-dependent CAT activity is shifted ~100-fold to the right in HDAC6 knockdown *versus* wild-type cells. In addition to the right shift in the dose response, there is a decrease in the maximal induction of CAT activity. As noted previously, the wild-type and HDAC6 knockdown cells have the same levels of GR protein, and we do not know why the maximal transactivating activity is decreased. The decrease may indicate an action of HDAC6 on proteins other than hsp90 (*e.g.* coactivator proteins) involved in the hormone response.

DISCUSSION

Here, we have shown that specific depletion of HDAC6 renders glucocorticoid receptors in HEK 293T cells deficient in steroid binding activity (Fig. 1, *A* and *B*) and in stable heterocomplex assembly (Fig. 1*C*). Neither the level of the GR (Fig. 1) nor its intrinsic ability to be assembled into stable GR·hsp90 heterocomplexes with steroid binding activity are affected by HDAC6 knockdown (Fig. 2). Cytosol prepared from knockdown cells is deficient in its ability to assemble stable GR·hsp90 heterocomplexes (Fig. 3), but the assembly activity is restored to the level of wild-type cytosol by addition of purified rabbit hsp90 (Fig. 5*A*). This suggests that hsp90 is the only component of the multichaperone assembly machinery that is affected by depletion of HDAC6. Consistent with this, hsp90 purified from HDAC6 knockdown cells is deficient at stable GR·hsp90 heterocomplex assembly when it is the hsp90 component of a purified five-protein assembly system (Fig. 6*B*). The deficiency in stable assembly by hsp90 from knockdown cytosol is reversed by preincubating with HDAC6 (Fig. 7).

Taken together, three differences between HDAC6 knockdown and wild-type hsp90s suggest how acetylation affects hsp90 function. The acetylated hsp90 has decreased ATP-binding affinity (Fig. 6*A*), it has reduced p23 binding (15), and it is capable of dynamic, but not stable, GR·hsp90 heterocomplex assembly (Fig. 6*B*). A decreased affinity of hsp90 for ATP was also seen when cells were treated with the general HDAC inhibitor FK228 (4). The cochaperone p23 (reviewed in Ref. 27) binds specifically to the ATP-dependent conformation of hsp90 (28, 29), and to have an open steroid binding cleft, the receptor-bound hsp90 must assume its ATP-dependent conformation (30). The acetylated hsp90 in HDAC6 knockdown cells can act as a component of the chaperone machinery to open the steroid binding cleft of the GR, as shown in Fig. 4*B*, but the opening is transient because p23 does not bind to stabilize the GR·hsp90 complex. p23 itself binds dynamically in stabilizing GR·hsp90 complexes in the steroid binding form (19). It is possible that the acetylated hsp90 achieves a p23 binding conformation, but it is so transient that p23 binding does not occur. Alternatively, acetylation may affect an ATP binding site on hsp90 such that ATP binds only weakly and the chaperone never achieves a p23 binding conformation. hsp90 contains a unique "Bergerat fold" ATP binding site at the N terminus that is the binding site for the hsp90 inhibitor geldanamycin (31–33), and it contains another nucleotide binding site near its C terminus (34–36), but it is not known whether acetylation affects one or both of these sites to yield decreased binding affinity for ATP-agarose. Regardless of the mechanism for the decreased p23 binding, the acetylation of hsp90 renders the chaperone incapable of stable GR·hsp90 assembly.

The dynamic assembly seen with acetylated hsp90 occurs with deacetylated hsp90 when p23 is not present in a purified assembly system (19, 24). In the normal cell with HDAC6 activity, very little of the hsp90 is acetylated and in the low ATP-binding affinity state. In the HDAC6 knockdown cell, the majority of hsp90 is acetylated, but cytosols prepared from knockdown cells still have some deacetylated hsp90 with high ATP-binding affinity that is capable of stable GR·hsp90 heterocomplex assembly (Fig. 4). The HDAC6 knockdown cells contain very little HDAC6 protein (15), and it is possible that activity of other deacetylating enzymes accounts for the presence of some hsp90 deacetylating activity in cytosol prepared from knockdown cells. Nevertheless, it is clear that HDAC6 activity is critical for normal response to hormone.

The wild-type GR in HDAC6 knockdown cells behaves like the 548/549/551 triple mutant GR (26) in that the dose response for dexamethasone-dependent transactivation is shifted about two orders of magnitude to the right (Fig. 8) with respect to wild-type cells expressing a normal level of HDAC6. This is the phenotype expected if the sole difference in both cases is a more rapid disassembly of the receptor·hsp90 complex. At any instant, only a few of the receptors in the cell would have an open steroid binding cleft and be accessible to

steroid. Because of rapid disassembly of the receptor·hsp90 heterocomplex, the proportion of time that the steroid binding cleft is open in the cell is very short, and a high concentration of steroid must be present to ensure entry before hsp90 dissociates and the ligand binding cleft closes. Thus, the GR in HDAC6-deficient cells is capable of ligand binding, nuclear translocation, and gene activation, and the HDAC6 deficiency with resulting hsp90 acetylation yields a phenotype in which much higher concentrations of steroid are required to achieve the same effect.

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Histone deacetylase 6 regulates growth factor-induced actin remodeling and endocytosis

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Abstract

Histone deacetylase 6 (HDAC6) is a cytoplasmic deacetylase that uniquely catalyzes α-tubulin deacetylation and promotes cell motility. However, the mechanism underlying HDAC6-dependent cell migration and the role for microtubule acetylation in motility are not known. Here we show that HDAC6-induced global microtubule deacetylation was not sufficient to stimulate cell migration. Unexpectedly, in response to growth factor stimulation, HDAC6 underwent rapid translocation to actin membrane ruffles and subsequently became associated with macropinosomes, the vesicles for fluid-phase endocytosis. Supporting the importance of these associations, membrane ruffle formation, macropinocytosis and cell migration were all impaired in HDAC6-deficient cells. Conversely, elevated HDAC6 level promoted membrane ruffle formation with a concomitant increase in macropinocytosis and motility. In search for a HDAC6 target, we found that heat shock protein 90 (Hsp90), another prominent substrate of HDAC6, was also recruited to membrane ruffles and macropinosomes. Significantly, inhibition of Hsp90 activity suppressed membrane ruffling and cell migration, while expression an acetylation-resistant mutant, promoted ruffle formation. Our results uncover a surprising role of HDAC6 in actin remodeling-dependent processes and identify the actin cytoskeleton as an important target of HDAC6-regulated protein deacetylation.

Introduction

Proper cell migration is critical for normal development as well as physiological processes such as wound healing, whereas its deregulation is a key factor contributing to tumor invasion and metastasis. Cell migration is often initiated by extracellular growth factors that activate receptor tyrosine kinase-dependent signaling, leading to extensive actin remodeling and the formation of unique structures termed membrane ruffles (19). These hallmark structures are important for cell motility as well as for invasive migration of cells through the extracellular matrix (53). Elucidating the molecular network that regulates and connects actin remodeling to cell migration is therefore fundamentally important.

Rac1, a member of the Rho family small GTPases, plays a critical role in actin remodeling and motility (5). In response to growth factor stimulation, Rac1 activates WAVE and the Arp2/3 multiprotein complex, which induces branching of actin filaments, resulting in membrane ruffling at the leading edge (lamellipodia or peripheral ruffles) and at the dorsal surface (dorsal ruffles). The induction of membrane ruffles is uniquely accompanied by a specialized form of endocytosis, fluid phase endocytosis, also termed macropinocytosis (15, 41). Distinct from other forms of endocytosis, macropinocytosis is initiated when extended membrane ruffles fuse with the plasma membrane, which generates large endocytic vesicles (> 0.1 μm) termed macropinosomes (11, 54). Macropinocytosis provides an efficient route for taking up extracellular macromolecules and nutrients.

As it is the case for cell migration, macropinocytosis in most cell types is stimulated by growth factors (11, 54). In fact, macropinocytosis and directed cell

movement are often correlated. For example, oncogenic Ras, Src and phosphoinositide 3-kinase (PI3K) promote both macropinocytosis and motility (1, 25, 51, 55, 57). A constitutively active p21-activated kinase 1 (PAK1), a key effector for Rac1, stimulates actin membrane ruffle formation and macropinocytosis with concomitant increase in directed cell migration (15). Analysis of fibroblasts derived from WAVE2 knockout mice demonstrates deficiency in ruffle formation, macropinocytosis and motility [Suetsugu, 2003 #1166]. Together, these observations reveal that macropinocytosis and cell motility are functionally connected and are subjected to the control of growth factor and oncogenic signaling. However, unlike clathrin-dependent or caveola-mediated endocytosis, the regulation of macropinocytosis is still poorly understood.

We have previously shown that HDAC6, a cytoplasmic member of the histone deacetylase family, promotes growth factor-induced cell motility (23). This unexpected finding is in stark contrast to the well-established function of the HDAC family in histone acetylation-dependent chromatin remodeling and gene transcription (13). Over-expression of HDAC6 in fibroblasts results in two prominent phenotypes: global microtubule deacetylation and an increase in cell motility (23). Indeed, HDAC6 uniquely possesses a microtubule deacetylase activity (23, 32, 61). However, the mechanism by which HDAC6 regulates cell motility remains poorly understood. Although microtubule network is a critical element in cell migration, whether microtubule deacetylation controlled by HDAC6 is the primary factor to drive cell motility is not known. This has become an important issue, as α-tubulin is not the exclusive substrate for HDAC6. One prominent HDAC6 substrate recently identified is the molecular chaperone Hsp90. HDAC6-catalyzed deacetylation of Hsp90 is required for its full chaperone activity,

which has been shown to regulate multiple signaling pathways (30, 34, 35). Identifying the relevant substrate would be critical for elucidating how the protein deacetylase HDAC6 regulates cell motility and likely uncover novel mechanisms that connect growth factor signaling to cell migration machinery.

In this study, we present evidence that HDAC6-induced microtubule deacetylation is not sufficient to regulate cell migration. Rather, HDAC6 and its substrate Hsp90 regulate cell motility and macropinocytosis by promoting actin remodeling-dependent membrane ruffle formation. We show that in response to growth factor stimulation, both HDAC6 and Hsp90 are recruited to membrane ruffles and macropinosomes, and that functional HDAC6 and Hsp90 are required for efficient Rac1 activation, ruffle formation, macropinocytosis and cell motility. Our study identifies HDAC6 as a new regulatory component in growth factor-induced actin remodeling, macropinocytosis and cell migration.

Materials and Methods

Cell lines:

Mouse embryonic fibroblasts (MEFs) obtained from E14.5 wild-type and HDAC6 knockout embryos were immortalized by stably expressing dominant negative p53 mutant (pCMVDD) following the protocol as previously described (49). MEFs are maintained and propagated in DMEM (Invitrogen) and 10% FCS at 37°C with 5% CO_2. Stable MEF lines were created through retrovirus-mediated gene transfer using pPABE-puro plasmids containing full-length HDAC6, HDAC6-mut or HDAC6-ΔBUZ. These construct are either green fluorescent protein (GFP)-tagged or FLAG-tagged.

Antibodies and Reagents:

Anti-mHDAC6 antibody was generated by injecting rabbits with recombinant GST-mouse HDAC6 (amino acids 991-1149) followed by affinity purification. The following antibodies were also used in this study: anti-human HDAC6 (H-300, Santa Cruz Biotechnology Inc.), anti-GFP (Roche), anti-SirT2 (Cell Signaling), anti-Rac1 (BD Bioscience), anti-p150 ^{glued} (BD Bioscience), anti-acetyl-α-tubulin (from Dr. M. Yoshida, RIKEN and from Sigma-Aldrich for immunostaining), anti-tyrosinated tubulin (a kind gift from Dr. J. C. Bulinski, Columbia University) and anti-HSP90 (26). Anti-actin (AC-15), anti-FLAG (M2), anti-polyglutamylated tubulin, anti-transferrin antibodies, geldanamycin, transferrin-biotin and Extravidin-HRP were from Sigma-Aldrich. Platelet-derived growth factor-BB (PDGF-BB) was from Sigma-Aldrich or Upstate Cell Signaling Solutions. F-Actin was stained with phalloidin-Alexa Fluor 647, phalloidin-rhodamine, or with phalloidin-Alexa Fluor 488 (Molecular Probes). Dextran (70kd)-tetramethylrhodamine (lysine fixable, Molecular Probes), Dextran (70kD)-Texas Red

(Molecular Probes) and Dextran (70kD)-FITC powder (Fluka) were used in macropinocytosis assays. Transfection reagents were from Roche (FuGene 6) or from Invitrogen (Lipofectamine LTX).

Immunofluorescence Microscopy:

Immunostaining was performed essentially as described previously (16, 23). Briefly, MEFs were cultured on glass coverslips in serum-free medium for 15 hours, followed by treatment with PDGF-BB at 50 ng/ml. Cells were fixed in 4% paraformaldehyde-phosphate buffered saline (PBS) for 15 minutes at room temperature. In macropinocytosis assays, cells containing Dextran–loaded macropinosomes were fixed for at least 2 hours with freshly prepared 4% paraformaldehyde-PBS. Cells were examined on a spinning disk confocal microscope (Olympus ZX-70) equipped with ORCA ER CCD camera using 60X/1.4 NA oil objective.

Macropinocytosis Assay:

Macropinocytosis assays were performed as previously described (12) with some modifications. Briefly, serum-starved MEFs were stimulated with PDGF (50 ng/ml) in culture medium in the presence of 3.0 mg/ml Dextran (70kD)-FITC for 45 minutes (or 1.0 mg/ml Dextran (70kD)-Texas Red for 30 minutes). Cells were rinsed with pre-chilled PBS and detached by 0.05% trypsin-0.02% EDTA-Na_2 (GIBCO) at 4°C. Cells were then washed and re-suspended in 5 mg/ml BSA (Sigma-Aldrich)-PBS before analyzed by FACS. Signals from wortmannin-treated (100 nM, 30 minutes pretreatment) samples was subtracted to eliminate dextra update from non-macropinocytic endocytosis. Propidium iodide (Molecular Probes, 1 μg/ml) was used to exclude dead cells in FACS analysis.

Endocytosis of Transferrin-Biotin:

Wild-type and HDAC6 knockout MEFs were serum-starved for 1.5 hours in DMEM and the cells were then incubated with 25 μg/ml transferrin-biotin in 5 mg/ml BSA-DMEM at 25°C for different periods of time. Cells were rinsed with ice-cold PBS, washed once with ice-cold 2 N acetate-2 M NaCl solution (pH 2.5), and lysed in 1% Triton X-100-PBS. Transferrin-biotin in the cell lysates was analyzed by ELISA assays using goat anti-transferrin antibody-coated plates and Extravidin-HRP.

Rac1-GTP Pull-down Assay:

Rac1 activation assay was preformed as described (43, 44). Briefly, PDGF-BB treated MEFs were lysed in Rac1-GTP binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1% NP-40, 10% glycerol, Protease Inhibitor Cocktail (Sigma-Aldrich), 1 mM Na_3VO_4, 5 mM NaF and 1 mM DTT). The cell lysates were incubated with 30 μg immobilized GST-PAK1-CRIB for 1hr at 4°C. Bound Rac1-GTP was examined by immunoblotting using monoclonal anti-Rac1 antibody.

Results

HDAC6 catalyzed deacetylation of microtubules alone is not sufficient to support cell migration

Cell migration is driven and sustained by dynamic yet coordinated rearrangements of actin and microtubule structures (42, 58). The microtubule network is post-translationally modified by acetylation, which has been suggested to modify microtubule stability (32, 38, 56). We have previously demonstrated that ectopically expressed HDAC6 promotes NIH3T3 cell migration, with markedly decreased microtubule acetylation (23). However, it is unclear whether HDAC6 regulates cell migration solely through deacetylation of microtubules and whether microtubule deacetylation alone is sufficient to drive cell migration.

In order to investigate this issue, we have generated HDAC6 knockout (KO) mice. Genomic sequence covering exons 10-13 of HDAC6 was deleted by homologous recombination (Figure 1A). Figure 1B and 1C verified that HDAC6 was absent from tissues or MEFs derived from HDAC6 KO mice. Importantly, α-tubulin acetylation was dramatically elevated in HDAC6 deficient tissues and MEFs by about 10-fold (Figure 1C). Of note, the majority of α-tubulin from HDAC6-deficient MEFs migrated slower in SDS-PAGE gel than those isolated from wild-type MEFs (Figure 1 C), suggesting that most α-tubulin subunits are acetylated in the absence of HDAC6. In contrast, tubulin polyglutamylation and tyrosination, two other well-known posttranslational modifications of tubulin (17, 40), are not affected by HDAC6 deficiency (Figure 1C). In agreement with the biochemical data, immunofluorescent microscopy revealed that the entire microtubule network in HDAC6-null cells was stained positive for acetylated α–tubulin

(Figure 1D, d), while in wild-type cells only sub-populations of the microtubule filaments are positive (Figure 1D, b). The level of another putative microtubule deacetylase, SirT2 (36), was not affected in HDAC6 KO cells (Figure 2B), indicating that SirT2 cannot substitute HDAC6 in catalyzing α-tubulin deacetylation. Importantly, as shown in Figure 1E, loss of HDAC6 results in a defect in cell motility. These results unambiguously establish that HDAC6 is the major enzyme catalyzing the deacetylation of microtubules and it is required for efficient cell motility.

We next determined whether tubulin hyperacetylation is the key to the migration defect observed in the HDAC6-deficient cells. Specifically, we asked whether cell motility could be restored in HDAC6 KO MEFs if hyperacetylated microtubules were deacetylated. To this end, we stably reconstituted HDAC6 KO MEFs with the following HDAC6 constructs (Figure 2A): GFP-tagged wild-type HDAC6 (HDAC6-GFP), a catalytically inactive mutant (HDAC6-mut-GFP), and a mutant that lacks the C-terminal ubiquitin-binding BUZ domain (HDAC6-ΔBUZ-GFP). The BUZ domain, also known as PAZ and ZnF-UBP (22, 47), is essential for HDAC6 to process misfolded proteins and regulate Hsp90 activity (28, 30). As expected, re-expression of wild-type HDAC6-GFP but not HDAC6-mut-GFP reduced acetylated α-tubulin to the level of wild-type cells. Interestingly, HDAC6-ΔBUZ-GFP was also fully competent to deacetylate microtubules. Therefore, the BUZ domain is not required for HDAC6 deacetylase activity toward α-tubulin.

We next examined the motility behavior of the HDAC6-deficient MEFs and the reconstituted lines in Boyden chamber migration assays. As shown in Figure 2C, re-expression of wild-type HDAC6 restored cell migration whereas catalytically inactive

HDAC6-mut-GFP was unable to do so. Unexpectedly, the HDAC6-ΔBUZ-GFP mutant failed to promote cell migration despite its higher expression level (Figure 2B) and the ability to potently induce α-tubulin deacetylation. These results demonstrate that both deacetylase activity and the ubiquitin-binding BUZ domain of HDAC6 are required to promote cell migration. Furthermore, global deacetylation of microtubules *per se* is not sufficient to enhance cell motility.

HDAC6 regulates membrane ruffle formation

As microtubule deacetylation alone is not sufficient to promote cell migration, we searched for other potential effectors of HDAC6. We first focused on the actin cytoskeleton as it plays a critical role in cell migration, often in concert with the microtubule network with which HDAC6 interacts. Upon stimulation with PDGF-BB, cells quickly formed ruffles at both the dorsal surface (arrows in Figure 3A) and the periphery of the cells (arrow heads in Figure 3A, lower panel). Remarkably, we found that HDAC6 quickly translocated to these ruffles as revealed by antibodies against either the endogenous HDAC6 (Figure 3A, upper panel), or the ectopically expressed HDAC6 (Figure 3A, lower panel). This association of HDAC6 to actin membrane ruffles is specific, as HDAC6 did not colocalize with stress fibers (Figure 3A, open arrows). Furthermore, careful examination revealed that HDAC6 was also concentrated at F-actin spots (Figure 3A, open arrowheads in the insert) that have been proposed to serve as precursor structures for dorsal membrane ruffles (31). Thus, HDAC6 becomes rapidly and selectively concentrated at actin-enriched membrane ruffles in response to growth factor stimulation.

To determine if HDAC6 association with membrane ruffles is functionally significant, we asked whether HDAC6 regulates the formation of membrane ruffles. We focused on dorsal ruffles as they are prominent structures and can be unambiguously identified. As shown in Figure 3B, significantly fewer cells from the HDAC6 KO group formed circular dorsal ruffles than their wild-type counterparts in response to PDGF treatment, indicating that HDAC6 is required for efficient membrane ruffle formation. Importantly, this defect was rescued by stable expression of wild-type HDAC6-GFP. In contrast, neither HDAC6-mut-GFP nor HDAC6-ΔBUZ-GFP rescued the defect (Figure 3B). In conclusion, similar to cell motility (Figure 2C), HDAC6 requires both its deacetylase activity and BUZ domain to promote membrane ruffles formation.

To further assess the activity of HDAC6 in membrane ruffle formation and cell motility, we determined whether over-expression of HDAC6 in wild-type MEFs enhance membrane ruffle formation. As shown in Figure 3C and 3D, HDAC6-GFP, but not enzymatically inactive HDAC6-mut-GFP, indeed promoted both membrane ruffling and cell migration. To our surprise, in contrast to its behavior in HDAC6-deficient cells, over-expression of HDAC6-ΔBUZ-GFP in wild-type MEFs stimulated both ruffle formation and cell motility. The unexpected activity of the HDAC6-ΔBUZ mutant is likely caused by complex formation between HDAC6-ΔBUZ and endogenous HDAC6 (Figure 3E), which possibly targets HDAC6-ΔBUZ mutant to the relevant substrates. Together, these results establish that HDAC6 can promote growth factor-induced membrane ruffle formation and this activity is tightly correlated with its ability to promote cell motility.

HDAC6 regulates macropinocytosis

It has been suggested that membrane ruffling is functionally linked to macropinocytosis (54). In characterizing HDAC6 subcellular localization in response to PDGF stimulation, we observed an association of HDAC6 with large intracellular vesicles (arrows in Figure 4). Interestingly, these vesicles were often continuous with or in close vicinity to both peripheral (Figure 4A, a-d) and dorsal membrane ruffles (Figure 4A, a'-d'). We concluded that these vesicles were macropinosomes based on several criteria. First, these vesicles were typically larger than 0.2 micrometers in diameter and positive for fluorescent-labeled Dextran, which is taken up by cells via macropinocytotic pathway (Figure 4A, b and b'). Second, these vesicles were encased with F-actin, another hallmark of macropinosomes (Figure 4A, c and c'). Lastly, generation of these large Dextran-containing vesicles was abolished by treatment with PI3K inhibitors (supplemental figure 2), which prevents macropinosome formation (2, 6, 9).

The association of HDAC6 with membrane ruffles and macropinosomes prompted us to investigate whether HDAC6 indeed regulates macropinocytosis. To this end, wild-type and HDAC6-deficient MEFs were incubated with Dextran-FITC in the presence of PDGF, and the uptake of fluorescent Dextran was measured by fluorescence activated cell sorting (FACS) analysis. In comparison to their wild-type counterparts, HDAC6 KO cells took up markedly less fluorescent Dextran (Figure 4B). Importantly, re-expression of wild-type but not catalytically inactive HDAC6, nor the ΔBUZ mutant in HDAC6 KO cells significantly restored the uptake of Dextran (Figure 4C). These results demonstrate that HDAC6 is required for efficient macropinocytosis. In contrast, the constitutive internalization of transferrin via the clathrin-dependent endocytosis is

comparable in wild-type and HDAC6-null cells (Figure 4D), suggesting HDAC6 specifically regulates macropinocytosis.

Hsp90 regulates membrane ruffle formation and cell migration

The finding that global microtubule deacetylation induced by HDAC6-ΔBUZ mutant is not sufficient to promote membrane ruffling, macropinocytosis and cell motility (Figure 2, 3 and 4) suggests that HDAC6 might work through additional substrates and mechanisms. One such candidate is Hsp90, a molecular chaperone implicated in many signaling cascades (35). To test this hypothesis, we first examined the subcellular distribution of Hsp90 by immunofluorescent microscopy. Hsp90 is largely present in the cytoplasm without a distinct pattern. However, upon PDGF treatment, Hsp90 also translocated to membrane ruffles where it colocalized with HDAC6 and actin (Figure 5A, a-f, close arrow heads). Similar to HDAC6, Hsp90 was also found concentrated at punctate F-actin structures (open arrowheads in Figure 5A, d-f) and nascent macropinosomes (open arrowheads in Figure 5A, g-i), but not at stress fibers. To determine whether Hsp90 could be functionally linked to those actin reorganization-associated cellular activities, we utilized geldanamycin, a specific Hsp90 inhibitor (52). As shown in Figure 5B and 5C, a brief treatment with geldanamycin significantly reduced PDGF-activated dorsal ruffle formation and motility in wild-type MEFs. Consistent with our findings that hyperacetylated Hsp90 retains some chaperone activity **(cite Kovacs and Murphy),** geldanamycin treatment can further reduce actin ruffle formation in HDAC6-null cells (supplemental figure 3). Together, these findings show that Hsp90, similar to HDAC6, is required for growth factor-induced membrane ruffling and cell migration.

To further investigate whether Hsp90 acetylation plays an important role in membrane ruffle formation, we utilized two Hsp90 mutants with mutations at lysine 294 which has been recently identified as a critical acetylated residue in Hsp90α (46). Toward this end, we transfected HDAC6 KO MEFs with expression plasmids for FLAG-tagged Hsp90α-WT, Hsp90α-K294R (acetylation-resistant) and Hsp90α-K294Q (acetylation-mimicking) and assessed membrane ruffle formation. As shown in Figure 5D, expression of Hsp90α-K294R markedly increased membrane ruffles, while the acetylation-mimicking Hsp90α-K294Q had no effect. The expression of Hsp90α-WT increased ruffle formation very slightly. These results strongly support the idea that deacetylation of K294 is important for Hsp90α to regulate actin remodeling.

HDAC6 and Hsp90 regulate Rac1 activity

Rac1 is a critical regulatory factor in ruffle formation and cell migration (20, 37, 39, 59). Similar to HDAC6 and Hsp90, Rac1 also translocated to membrane ruffles (Figure 6A, a-c, arrowheads) and F-actin dots (open arrowheads), which strongly suggested the possibility that HDAC6 and Hsp90 might regulate Rac1 activity. To examine Rac1 activation in wild-type and HDAC6 KO cells. We measured the binding of intracellular Rac1-GTP (the active form of Rac1) to GST-PAK1-CRIB (Cdc42/Rac interactive binding domain (44)). As shown in Figure 6B, after PDGF stimulation, Rac1 from wild-type cells was activated and able to associate with GST-PAK1-CRIB. In contrast, Rac1 activation was markedly reduced in HDAC6 KO MEFs. Consistent with the hypothesis that HDAC6 functions in conjunction with Hsp90, treatment of cells with geldanamycin also significantly reduced the activation of Rac1 (Figure 6C). Taken together, these data support the idea that HDAC6 and Hsp90 are required for full

activation of Rac1, and consequently for efficient membrane ruffle formation and cell migration.

Discussion

The identification of HDAC6 as a microtubule-associated deacetylase has revealed an unexpected link between HDAC-regulated protein acetylation and biological processes independent of histone modification (Hubbert et al., 2002). In this report we have discovered a surprising activity of HDAC6 in the regulation of membrane ruffle formation, macropinocytosis and cell motility, thus identifying HDAC6 as a new regulatory component in actin remodeling.

Microtubule acetylation has been observed for more than twenty years; however, its functional significance remains poorly understood. The dramatic increase in α-tubulin acetylation observed in HDAC6 knockout (KO) brain tissue and MEFs provides the definitive proof that HDAC6 is a tubulin deacetylase *in vivo*. Remarkably, α-tubulin from HDAC6 KO MEFs migrates at a slower mobility than that from wild type cells, which could be caused by an increase in the molecular weight and change in the overall charge of α-tubulin by acetylation. This finding strongly indicates that the majority of α-tubulin population has become acetylated in the absence of HDAC6 Our findings demonstrate that HDAC6 is the dominant tubulin deacetylase *in vivo* and indicate that other putative tubulin deacetylases, such as SirT2, would likely play little role in microtubule acetylation. Indeed, immuno-staining analyses showed that the entire microtubule network becomes acetylated in HDAC6 deficient MEFs. ~~Surprisingly, this hyperacetylated microtubule network is capable of performing all essential microtubule functions as HDAC6 deficient cells and mice are viable (this study). However,~~ Consistent with previous findings that α-tubulin acetylation is often associated with more stable microtubule population (REF), microtubules in HDAC6 deficient cells are indeed more

stable and less dynamic (REF). This increase in microtubule acetylation has been proposed to affect the formation of adhesion complexes (JCS ref). ~~Whether microtubule acetylation affects other important function of microtubules, such as migration, is a critical issue to be addressed.~~

HDAC6 has been shown to regulate cell migration in response to serum growth factors (23). Over-expression of HDAC6 promotes while pharmacological or genetic inactivation of HDAC6 inhibits cell motility ((18, 23) and Figure 1 and 2). As HDAC6-dependent cell motility is tightly correlated with the acetylation status of α-tubulin, the first substrate identified for HDAC6, we have speculated that microtubule acetylation is critical for cell motility. Surprisingly, our data clearly show that tubulin deacetylation and cell migration can be uncoupled. Although the BUZ domain-deleted HDAC6 mutant potently catalyzes α-tubulin deacetylation, thus likely resulting in decreased microtubule stability and increased microtubule dynamics, it cannot promote cell migration (Figure 2). Therefore, global deacetylation of tubulin is not sufficient to promote cell migration. Instead, several lines of evidence in this study identify the actin cytoskeleton as a main target of HDAC6 in cell motility. We found that HDAC6 becomes rapidly associated with the actin cytoskeleton in response to growth factor stimulation, concentrating at F-actin spots, membrane ruffles and macropinosomes (Figure 3 and 4). These dynamic structures have long been suggested to associate with cell motility (37, 39). Supporting a critical function of HDAC6 in these actin-remodeling processes, we showed that cells deficient in HDAC6 are defective in membrane ruffle formation, macropinocytosis and cell migration. Conversely, over-expression of wild-type HDAC6 promotes actin membrane ruffle formation, macropinocytosis and cell motility (Figure 2, 3 and 4). These

findings demonstrate a regulatory role for HDAC6 in growth factor-induced actin remodeling, and provide a molecular mechanism by which HDAC6 controls cell motility.

Mechanistically, we found that Hsp90 and Rac1 are also concentrated at actin-associated membrane ruffles (Figure 5 and 6). Thus, HDAC6 and its substrate Hsp90, as well as Rac1, the key factor in actin remodeling machinery, become co-localized in response to growth factor stimulation. These observations suggest the interesting possibility that HDAC6 might affect Rac1 activation (Figure 6B) through regulation of Hsp90, whose full chaperone activity requires HDAC6 (4, 30, 34). Consistent with this notion, inactivation of either HDAC6 or Hsp90 dampens Rac1 activation (Figure 6), resulting in defective membrane ruffling and cell motility (Figure 2, 3 and 5). Although there are likely additional targets of HDAC6 in the actin remodeling machinery, our study suggests that HDAC6 contributes to actin remodeling, at least in part, by regulating Hsp90. This idea is further supported by our finding that acetylation-resistant Hsp90-K294R but not acetylation-mimicking K294Q mutant could partially rescue the membrane ruffle defect in HDAC6 deficient MEFs (Figure 5D). This conclusion is also consistent with the observation that HDAC6 catalytic inactive mutant and ΔBUZ mutant, which cannot bind and regulate Hsp90{Kovacs, 2005 #12}, are also deficient in supporting actin remodeling (Figure 2C, 3C and 4C). Despite their inability to efficiently promote actin remodeling, interestingly, both mutants can still associate with membrane ruffles upon growth factor stimulation. This finding indicates that the recruitment to actin-dependent structure per se is not sufficient for HDAC6 to function in actin remodeling.

Our study shows that HDAC6 is involved in the regulation of macropinocytosis. Of all forms of endocytosis, macropinocytosis is the least understood but tightly linked to growth factor and oncogenic signaling ((1, 27, 54, 57). In fact, induction of macropinosome formation is one of the first cellular effects observed for the oncogenic ras (5). In contrast to normal cells, macropinocytosis is commonly deregulated and becomes constitutive in tumor cells. Deregulated macropinocytosis might enhance motility and facilitate uptake of nutrients for aggressive tumor growth. Our finding that HDAC6 is required for efficient macropinocytosis (Figure 4) identifies HDAC6 as a novel regulator in this poorly characterized but important endocytic pathway. It is important to point out that both HDAC6 and actin are associated with nascent macropinosomes after they are internalized. This observation suggests that HDAC6 and the actin machinery might be involved in the intracellular trafficking of the vesicles. This is an intriguing possibility in light of our previous observation that HDAC6 associates with dynein motors and has the capacity to regulate protein transport (28). Interestingly, the ability of HDAC6 to regulate both protein transport and macropinocytosis (Figure 4) requires the ubiquitin-binding BUZ domain, suggesting that HDAC6 regulates endocytosis through binding to ubiquitins or ubiquitinated proteins. Given the prominent role of ubiquitin modification in endocytic trafficking (21, 33), we speculate that HDAC6 might play additional roles in trafficking of the endocytic vesicles, thereby further affecting growth factor signaling and cell motility.

Our results uncover an important function for HDAC6 in signaling-dependent actin remodeling. It is worth noting that HDAC6 has been implicated in the formation of mDia-dependent podosomal ring (7, 14), a F-actin-enriched structure (8). HDAC6 was

also reported to be present at another F-actin structure, the T cell immunological synapse (48). Although those studies did not address a specific role for HDAC6 in podosomes or immune synapse formation, they are nonetheless consistent with the proposition that HDAC6 likely plays a role in a broad range of actin reorganization-dependent processes. It is important to point out that HDAC6 is not an essential gene. HDAC6 knockout mice are viable and fertile but show modest behavioral abnormality (Y.K. and T.P.Y. unpublished observation). The lack of prominent defects in animals deficient in HDAC6 might reflect the fact that actin reorganization and cell migration are redundantly controlled by multiple pathways under normal conditions (5). Nevertheless, given that oncogenic Ras, Src and PI3K can all potently induce membrane ruffling, macropinocytosis and cell migration (25, 51, 55), our findings underline the potential importance of HDAC6 in tumor development and metastasis. In this regard, inhibitors for HDACs are potent anti-tumor agents. Although their anti-tumor activities have been mostly attributed to the regulation of gene expression, our findings that HDAC6 is capable of regulating cell migration and endocytosis suggest HDAC inhibitors could potentially elicit anti-tumor activity via a non-genomic mechanism.

Extensive work on histone deacetylases has firmly established their roles in chromatin remodeling and transcriptional regulation (13). The recent identification of a large number of non-histone substrates, however, implicates novel functions for the HDAC family in previously unexpected areas (10, 29). Our characterization of HDAC6 as an important regulatory factor in membrane ruffle formation, endocytosis and cell migration is another surprising addition to the activities of HDACs and suggests an important role for reversible acetylation in actin remodeling-dependent processes.

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Figure Legends

Figure 1. Accumulation of hyperacetylated microtubules in HDAC6 knockout tissues and MEFs. (A) A schematic diagram of the targeting strategy with mouse HDAC6 genomic sequences. Exons (vertical bars) 10-13 were replaced by a targeting vector containing a Neo and Zeo cassette, resulting in the disruption of the first catalytic domain. The sequences are not drawn to the scale. **(B-C)** Immunoblotting analysis of brain samples (B) or MEFs (C) from wild-type and HDAC6 knockout (KO) mice revealed the absence of HDAC6 protein (upper panel) and significantly elevated acetylated tubulin in HDAC6 knockout animals (middle panel). The relative amount of acetylated tubulin was provided under Panel C. $p150^{glued}$ was used as a loading control. **(D)** Wild-type (a, b) and HDAC6 KO (c, d) MEFs were immuno-stained with antibodies for acetylated-α-tubulin (red) and anti-α-tubulin (green). Note that almost the entire microtubule network in HDAC6 KO MEFs stains positive for acetylated α-tubulin. Bars = 10 μm. **(E)** Cell motility of wild-type and KO MEFs was analyzed by Boyden chamber cell migration assays. Average values with standard error of the mean (SEM) from 4 independent experiments were shown.

Figure 2. Both enzymatic activity and BUZ domain of HDAC6 are required to rescue motility defects of HDAC6 knockout cells. **(A)** A schematic representation of the human HDAC6 constructs used in this study. The constructs are either tagged with GFP or with FLAG. Boxes in black: catalytical domains; Boxes in grey: BUZ domain. H216A and H611A are histidine to alanine point mutations that inactivate the deacetylase activity of HDAC6. **(B)** Wild-type, HDAC6 knockout, and HDAC6 knockout MEFs stably expressing the human HDAC6 constructs were analyzed for the level of HDAC6 in the

cells using an anti-human HDAC6 antibody (upper panel). In addition, HDAC6-ΔBUZ-GFP sample was also serially diluted by 2 and 4 fold for analysis. Total and acetylated α-tubulin and SirT2 levels were examined using corresponding antibodies. **(C)** Quantification of cell migration using Boyden chamber assays for cell lines described in (B). Each bar represents average value + SEM from 4 independent experiments. *: $t < 0.05$ in 2-tailed and paired *t*-test.

Figure 3. HDAC6 translocates to actin membrane ruffles and is required for efficient ruffle formation. **(A)** Wild-type (upper panel) or HDAC6 knockout (KO) MEFs that stably express human HDAC6-FLAG (lower panel) were stimulated with PDGF-BB (50 ng/ml for 8 minutes) and stained with an antibody for HDAC6 or FLAG and phalloidin-Alexa Fluor-488 for F-actin (green). Endogenous HDAC6 (red, upper panel) and FLAG-tagged HDAC6 (red, lower panel) are both detected at dorsal (close arrows) and peripheral (close arrow heads, lower panel) membrane ruffles. Open arrows indicate stress fibers. Insert in upper panel: An enlarged image shows the concentration of HDAC6 at actin spots (open arrowhead). Bars = 10 μm. **(B)** Quantification of PDGF-BB-induced dorsal ruffle formation in cell types indicated. Graph represents cells with dorsal circular ruffles. Approximately 500 cells were examined from each group. Results are averages + SEM. n = 5 for WT and KO cells; n = 4 for KO + reconstituted cells. **: $t < 0.01$ in 2-tailed *t*-test. **(C)** Wild-type MEFs that stably express GFP control, HDAC6-GFP, HDAC6-mut-GFP or HDAC6-ΔBUZ-GFP were examined for dorsal ruffle formation as in (B). Each bar represents average value + SEM from 4 independent experiments. **: $t < 0.01$ in a 2-tailed and paired *t*-test. **(D)** Cell lines described in (C) were tested in Boyden chamber migration assays and relative motility values are graphed.

Each bar represents the average value + standard deviation from 3 independent experiments with duplicate wells. *: $t < 0.05$ in 2-tailed and paired *t*-test. **(E)** 293T cells were co-transfected with HDAC6-ΔBUZ-FLAG and HDAC6-GFP and processed for immunoprecipitation using anti-FLAG, anti-GFP or control mouse antibody. The precipitates were analyzed by immunoblotting using anti-GFP (left panel) or anti-FLAG (right panel) antibody to show mutual association of HDAC6-ΔBUZ and the full-length HDAC6.

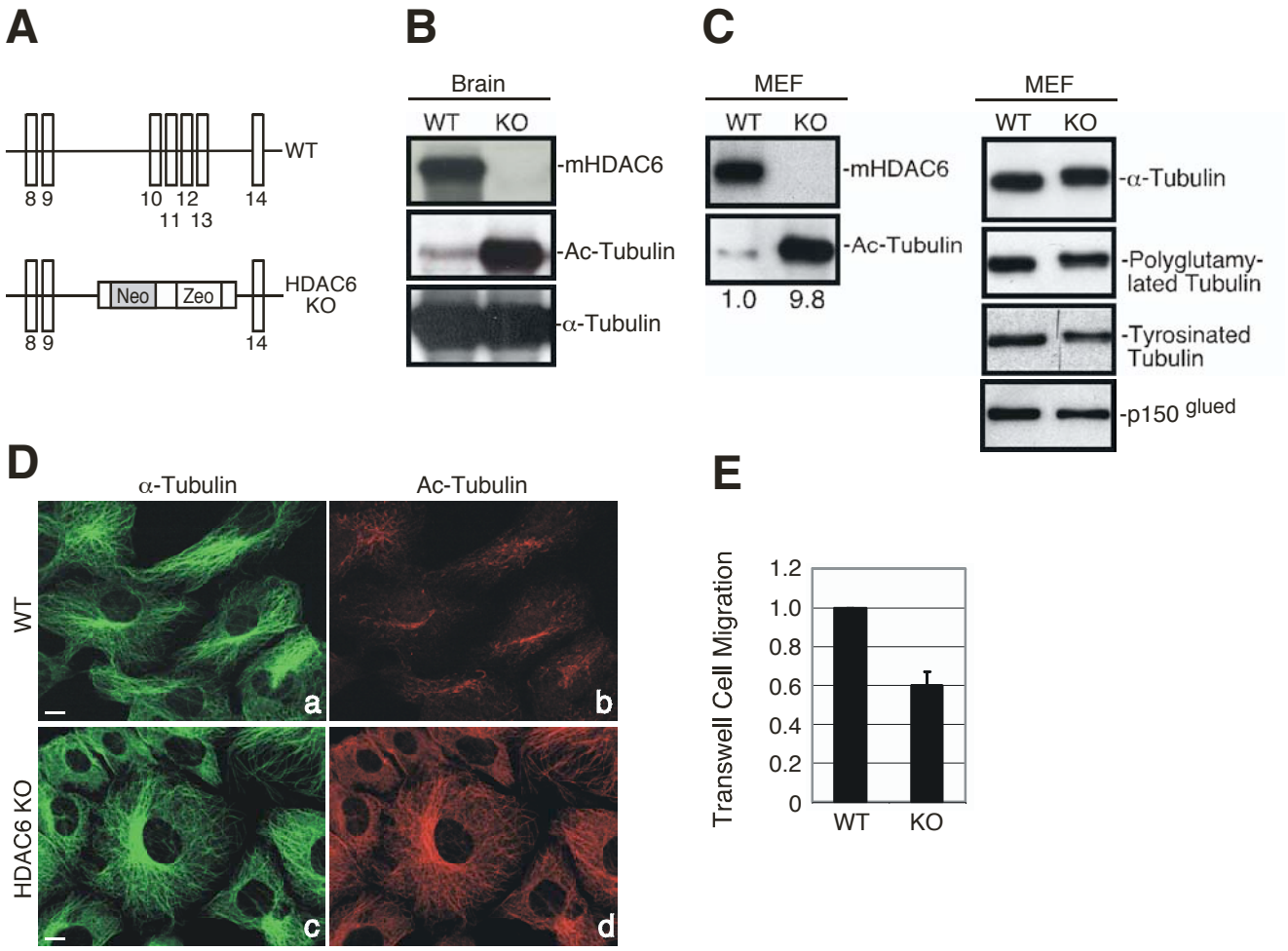
Figure 4. HDAC6 regulates macropinocytosis. **(A)** Dorsal ruffle formation and macropinocytotic uptake was visualized in HDAC6 knockout MEFs that stably express human HDAC6-FLAG. The cells were stimulated with 20 ng/ml PDGF-BB in the presence of 3 mg/ml Dextran (70kD)-tetramethylrhodamine for 10 minutes. Triple labeling using anti-hHDAC6 and phalloidin Alexa Fluor 647 revealed colocalization (arrows) of HDAC6 (green) and F-actin (blue) at the Dextran-positive macropinosomes (red). Bars = 10 μm. **(B)** Fluorocytometric analysis revealed a significant decrease in macropinocytosis of Dextran-FITC in HDAC6 KO MEFs. Values are averages + SEM from 4 independent experiments. **(C)** HDAC6 KO MEFs and KO cells stably expressing human HDAC6 constructs as indicated were assessed for the uptake of Dextran (70 kD)-Texas Red as described in (B). Values are averages + SEM from 4 independent experiments. *: $t < 0.05$ in 2-tailed and paired *t*-test. **(D)** Transferrin uptake remained unaffected in HDAC6 knockout MEFs. Biotinylated transferring was incubated with wild-type or KO MEFs and internalized transferring at different time points was measured as described in Material and Method. Average values from 2 independent experiments with quadruple wells are shown.

Figure 5. Hsp90 regulates membrane ruffle formation and cell migration. **(A)** Colocalization of Hsp90 (b, e, and h) with HDAC6 (a) or F-actin (d, g) in wild-type MEFs stimulated with 50 ng/ml PDGF-BB for 8 minutes. Close arrowheads indicate ruffles. Open arrowheads in d-f point to Hsp90 (red)-containing F-actin (green) spots. Open arrowheads in g-i indicate colocalization of Hsp90 (red) and F-actin (green) at the macropinosomes. Bars = 10 μm. **(B)** Inhibition of PDGF-induced dorsal ruffle formation in wild-type MEFs was dose-responsive to geldanamycin treatment (2 hours at 3 μM or 10 μM). Bars represent average values + SEM. n=4. **(C)** Cells pretreated with either vehicle or geldanamycin at 1 μM or 5 μM for 2 hours were analyzed for motility in Boyden chamber migration assays. The average value from 2 independent experiments with duplicate wells is shown. Note that geldanamycin treatment did not induce cytotoxicity under these experimental conditions. **(D)** HDAC6 KO MEFs were transfected with FLAG-tagged wild-type, mutant human Hsp90α constructs or a control plasmid as indicated. Cells were stimulated 50 ng/ml PDGF-BB and stained with phalloidin-rhodamine and anti-FLAG to identify membrane ruffles and transfected cells respectively. The number of cells that formed dorsal ruffles were scored (Average value + SEM). At least 400 FLAG-positive and 800 non-transfected cells on the same cover glass were examined in four independent experiments. Dotted line indicates the level of ruffle formation in non-transfected cells. **: $t < 0.01$ in a 2-tailed *t*-test.

Figure 6. Functional HDAC6 and Hsp90 are required for efficient Rac1 activation. **(A)** Wild-type MEFs were stimulated with 50 ng/ml PDGF-BB for 8 minutes. Colocalization of Rac1 (b) with F-actin (a) is observed at dorsal ruffle (close arrowheads) and F-actin dots (open arrowheads) using anti-Rac1 antibody and phalloidin-Alexa Fluor-488. Bar =

10 μm. **(B)** Rac1 activation in wild-type and HDAC6 knockout MEFs was evaluated by GST-PAK1-CRIB pull-down assay. A diminished Rac1 activation in response to PDGF-BB treatment was observed in HDAC6 KO MEFs. **(C)** Pretreatment of wild-type MEFs with vehicle control, wortmannin (100 nM for 30 minutes as another control) or geldanamycin at indicated concentrations for 2 hours led to reduced Rac1 activation by PDGF-BB treatment (50 ng/ml for 10 minutes) in a GST-PAK1-CRIB pull-down assay. Note that wortmannin treatment inhibits PI3K function and Rac1 activation (24, 45).

figure 1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Ac-Tubulin
WT
HDAC6 KO
a
b
c
d
E
Transwell Cell Migration
1.2
1.0
0.8
0.6
0.4
0.2
0
WT KO

figure 2

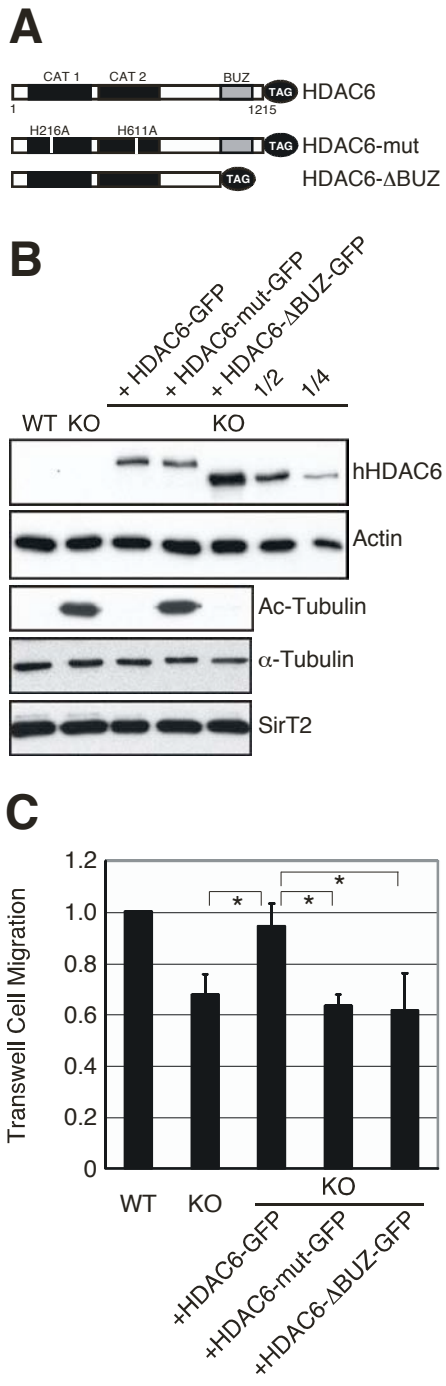

A
CAT 1
CAT 2
BUZ
TAG
HDAC6
1
1215
H216A
H611A
HDAC6-mut
HDAC6-ΔBUZ
B
+ HDAC6-GFP
+ HDAC6-mut-GFP
+ HDAC6-ΔBUZ-GFP
1/2
1/4
WT
KO
KO
hHDAC6
Actin
Ac-Tubulin
α-Tubulin
SirT2
C
Transwell Cell Migration
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
*
WT
KO
KO
+HDAC6-GFP
+HDAC6-mut-GFP
+HDAC6-ΔBUZ-GFP

figure 3

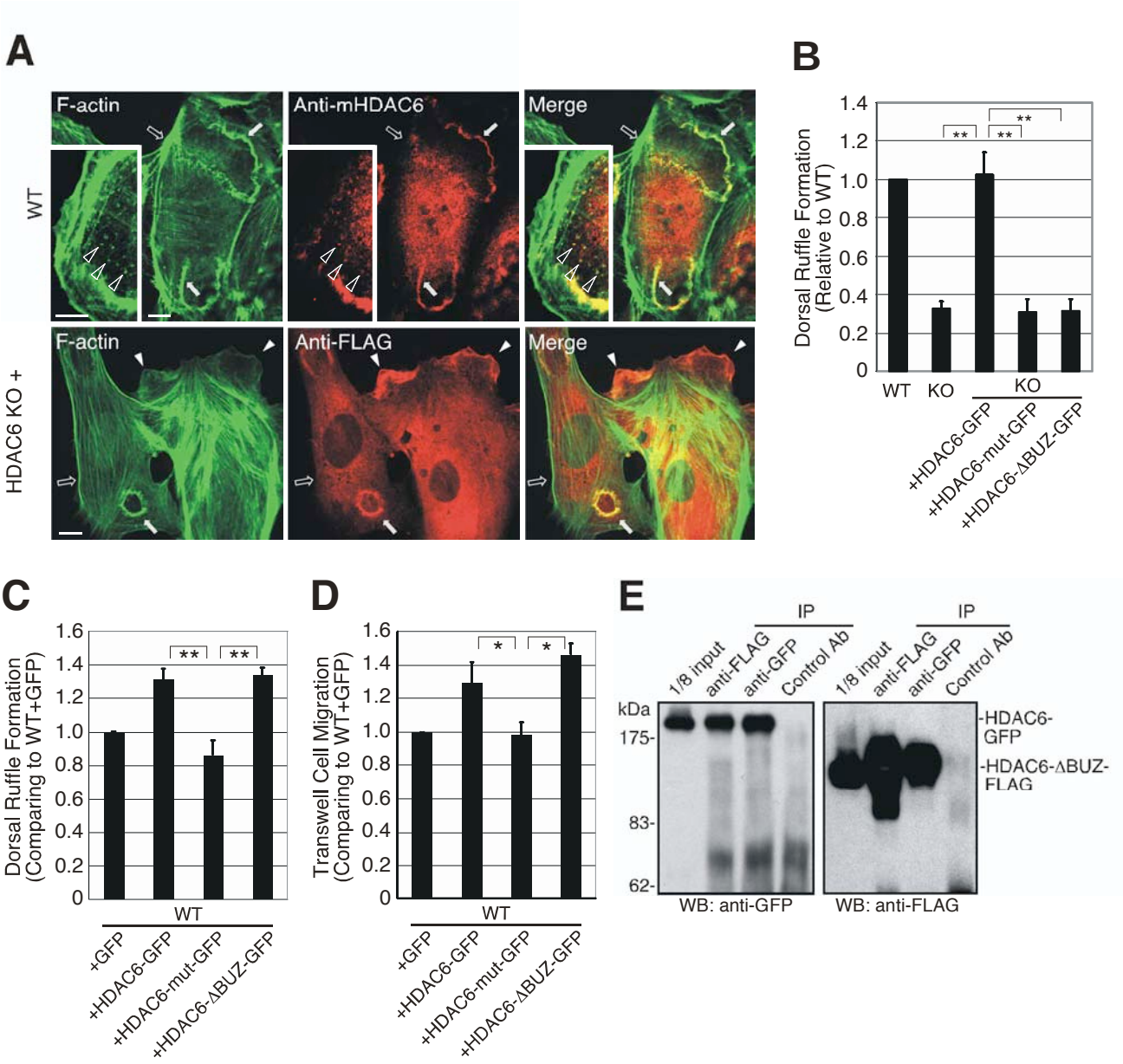

A
F-actin
Anti-mHDAC6
Merge
WT
HDAC6 KO +
F-actin
Anti-FLAG
Merge
B
Dorsal Ruffle Formation (Relative to WT)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
**
WT
KO
KO
+HDAC6-GFP
+HDAC6-mut-GFP
+HDAC6-ΔBUZ-GFP
C
Dorsal Ruffle Formation (Comparing to WT+GFP)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
WT
+GFP
+HDAC6-GFP
+HDAC6-mut-GFP
+HDAC6-ΔBUZ-GFP
D
Transwell Cell Migration (Comparing to WT+GFP)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
WT
+GFP
+HDAC6-GFP
+HDAC6-mut-GFP
+HDAC6-ΔBUZ-GFP
E
IP
IP
1/8 input
anti-FLAG
anti-GFP
Control Ab
1/8 input
anti-FLAG
anti-GFP
Control Ab
kDa
175-
83-
62-
-HDAC6-GFP
-HDAC6-ΔBUZ-FLAG
WB: anti-GFP
WB: anti-FLAG

figure 4

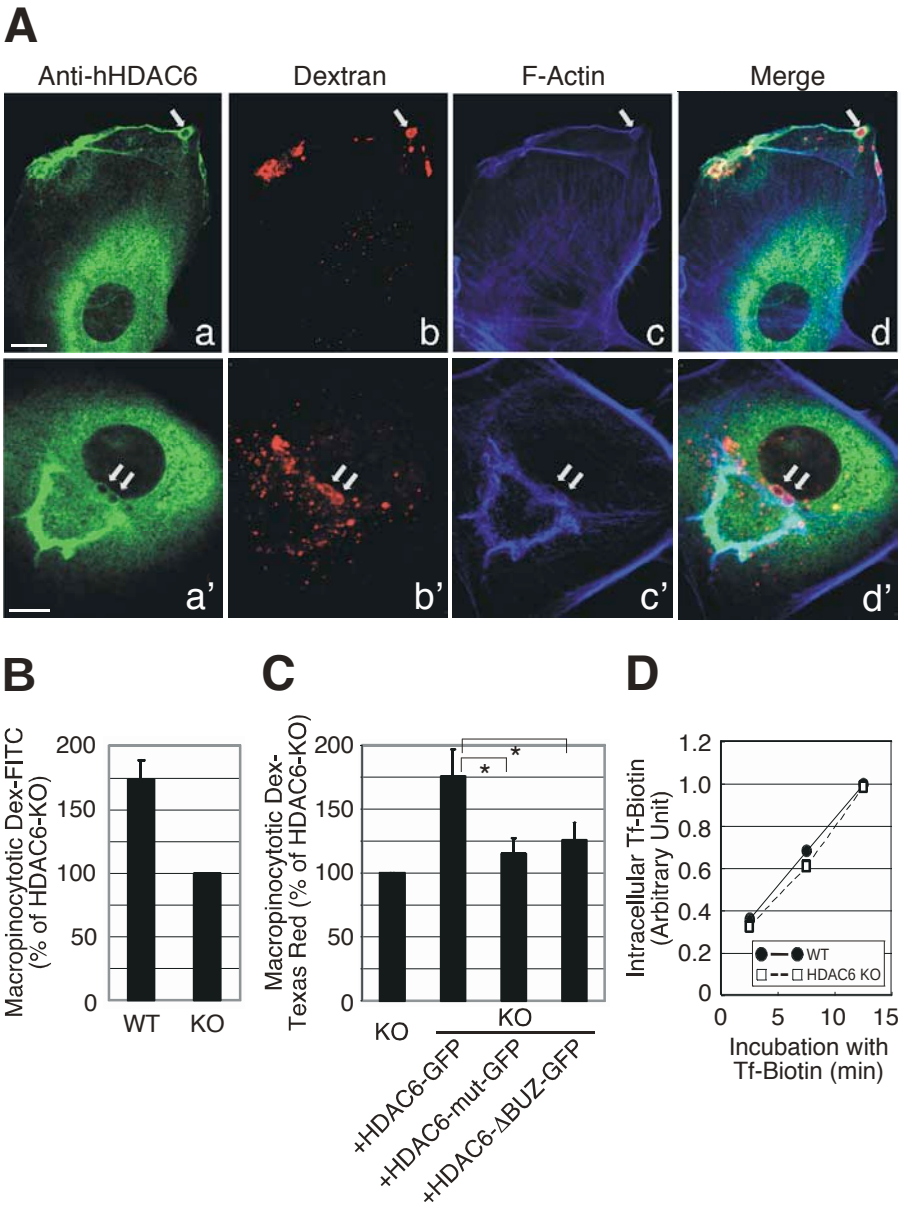

A
Anti-hHDAC6
Dextran
F-Actin
Merge
a
b
c
d
a'
b'
c'
d'
B
Macropinocytotic Dex-FITC (% of HDAC6-KO)
200
150
100
50
0
WT
KO
C
Macropinocytotic Dex-Texas Red (% of HDAC6-KO)
200
150
100
50
0
*
*
KO
KO
+HDAC6-GFP
+HDAC6-mut-GFP
+HDAC6-ΔBUZ-GFP
D
Intracellular Tf-Biotin (Arbitrary Unit)
1.2
1.0
0.8
0.6
0.4
0.2
0
WT
HDAC6 KO
0
5
10
15
Incubation with Tf-Biotin (min)

figure 6

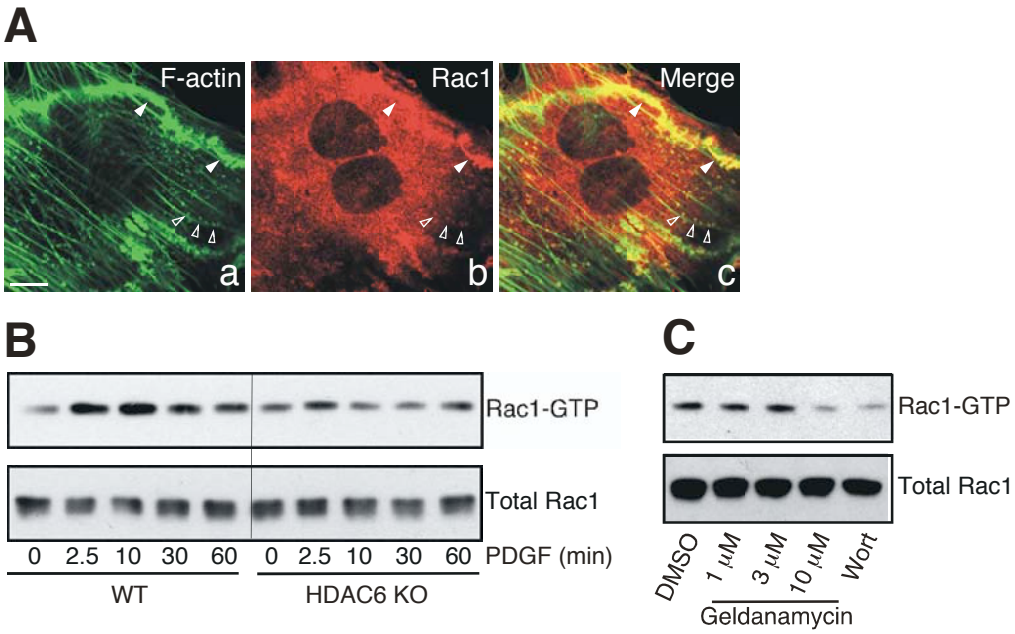
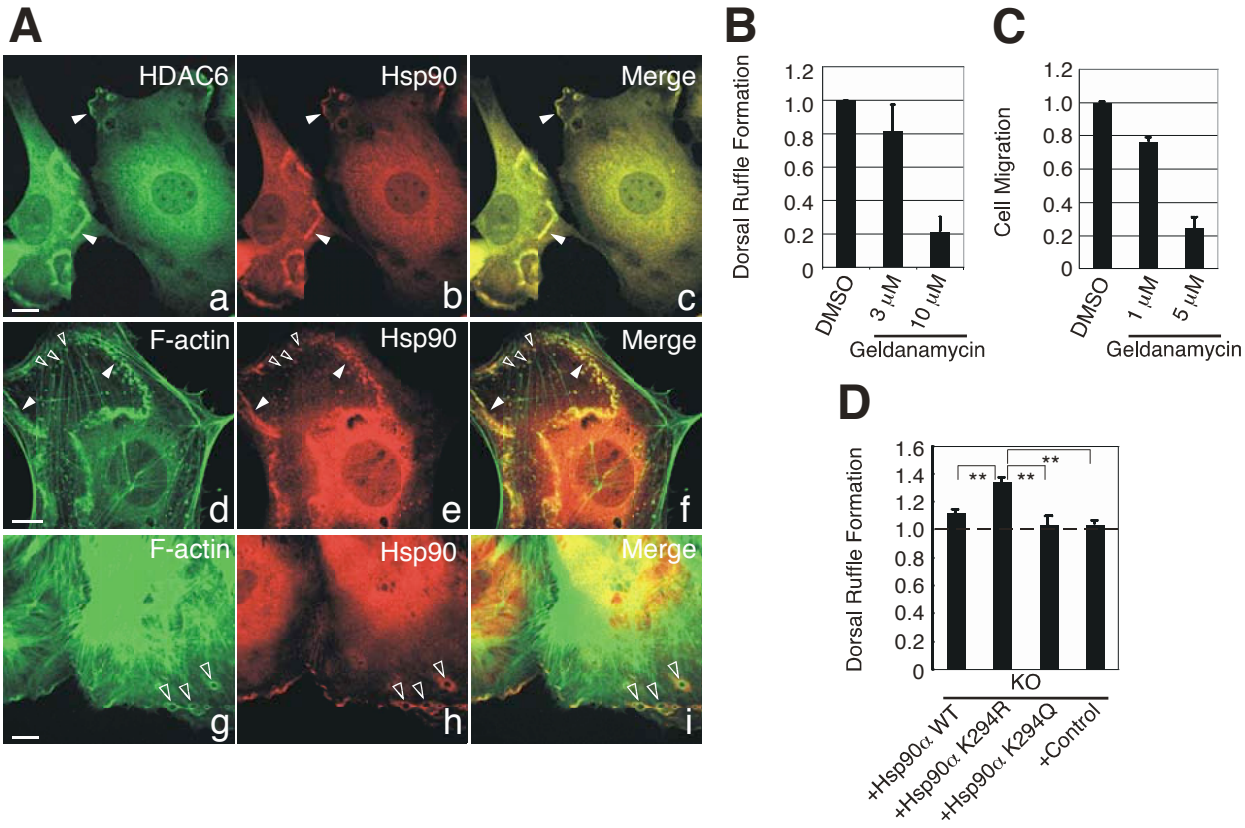

A
F-actin
Rac1
Merge
a
b
c
B
Rac1-GTP
Total Rac1
0 2.5 10 30 60 0 2.5 10 30 60 PDGF (min)
WT
HDAC6 KO
C
Rac1-GTP
Total Rac1
DMSO
1 μM
3 μM
10 μM
Wort
Geldanamycin

figure 5


A
HDAC6
Hsp90
Merge
a
b
c
F-actin
Hsp90
Merge
d
e
f
F-actin
Hsp90
Merge
g
h
i
B
Dorsal Ruffle Formation
1.2
1.0
0.8
0.6
0.4
0.2
0
DMSO
3 μM
10 μM
Geldanamycin
C
Cell Migration
1.2
1.0
0.8
0.6
0.4
0.2
0
DMSO
1 μM
5 μM
Geldanamycin
D
Dorsal Ruffle Formation
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
**
KO
+Hsp90α WT
+Hsp90α K294R
+Hsp90α K294Q
+Control